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(57) Abstract

The present invention relates to a protein, termed t-complex associated testes expressed-1 (tcte-1) that is found on the surface of mature sperm and is responsible for the binding of sperm to egg during mammalian fertilization. The invention provides for tcte-1 nucleic acids, proteins, antibodies directed toward tcte-1, vaccines, and methods of immunocontraception.

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SPERM SURFACE PROTEIN

1. INTRODUCTION

The present invention relates to a protein, termed t-complex associated testes expressed-1 (tcte-1), that is found on the surface of mature sperm and is responsible for the binding of sperm to egg during mammalian fertilization. It is based, at least in part, on the cloning and characterization of human and mouse cDNAs that encode tcte-1 protein.

2. BACKGROUND OF THE INVENTION

2.1. THE BIOLOGY OF FERTILIZATION

The process of fertilization comprises a number of steps which occur in a compulsory order (Wassarman, 1987, Science 235:553-560). These steps, performed in the proper sequence, provide for species specific recognition between an egg and a single sperm, thereby avoiding the pitfalls of interspecies hybrids and polyploidy.

The first step toward fertilization occurs when free swimming sperm form a relatively loose, nonspecific association with an ovulated egg at the surface of the egg's thick extracellular coat, called the zona pellucida.

Next, attached sperm form a more tenacious, species-specific association with the egg. This step, called binding, is mediated by receptors present in the zona pellucida which interact with complementary egg-binding proteins present in sperm plasma membrane.

After binding to the egg, a sperm releases a number of enzymes that enable it to tunnel through the zona pellucida to reach the plasma membrane of the egg. These enzymes, which include proteinases, glycosidases, phosphatases, arylsulfatases, and phospholipases, are stored in a membrane-bound,

lysosome-like organelle called the acrosome that occupies the anterior region of the sperm head. The release of enzymes, called the acrosome reaction, involves fusion of the outer acrosomal membrane and sperm plasma membrane at many sites, and results in the release of small, hybrid vesicles from the anterior region of the sperm head.

Acrosome-reacted sperm are then able to penetrate the zona pellucida, burrowing at a rate of about one micrometer per minute and creating a tunnel little wider than the sperm head.

Once a sperm reaches the space between the zona pellucida and the egg plasma membrane, it is able to fuse with the egg, thereby achieving fertilization. Sperm that subsequently reach the egg plasma membrane are prevented from fusing with the egg by rapid depolarization of the egg membrane. Fertilization by additional sperm ("polyspermy") is further avoided by changes in the zona pellucida which render it impenetrable to bound sperm and prevent other sperm from binding. These changes in the zona pellucida, generally referred to as the zona reaction, are effected by the release of enzymes from cortical granules located just beneath the egg plasma membrane in a process called the cortical reaction.

2.2. SPERM SURFACE PROTEINS

In sea urchins, the tight binding between sperm and egg is mediated by bindin, a protein found in the sperm inner acrosomal membrane (Glabe et al., 1982, J. Cell Biol. 94:123; Vacquier and Moy, 1978, Proc. Natl. Acad. Sci. U.S.A. 74:2456) which is only available for binding after the acrosome reaction has occurred. This differs from mammalian fertilization, in which

sperm which have undergone the acrosome reaction are incapable of binding to eggs.

A number of mammalian sperm surface proteins are currently being considered as possible analogs of bindin in the egg-binding process. These include the following:

Bleil and Wasserman (1990, Dev. Biol. 87:5563-5567) have identified a 56,000 dalton protein on mouse sperm that binds ZP3, an 83,000 dalton glycoprotein found at the surface of the zona pellucida of mouse eggs that appears to serve as the primary receptor for acrosome intact sperm. This 56,000 dalton protein was found to be heavily radiolabeled by photoaffinity crosslinking of sperm with ZP3 conjugated to ^{125}I-labeled photoactivatable heterobifunctional crosslinker, and was observed to bind very tightly to ZP3-affinity columns.

PH-20 is an approximately 60,000 dalton guinea pig sperm membrane protein which appears to exist in two distinct populations (Carron and Saling, in "Elements of Mammalian Fertilization", Volume II, Wassarman, ed., CRC Press, Boston, pp. 147-176). In cauda epididymal sperm, PH-20 expression was found to be confined to the surface of the post-acrosomal segment and the luminal surface of the inner acrosomal membrane (Phelps and Myles, 1987, Dev. Biol. 123:63). These two populations have been designated PH-20$_{pm}$ and PH-20$_{am}$, respectively, with PH-20$_{am}$ accounting for about seventy percent of the total (Cowan et al., 1987, J. Cell Biol. 103:1289). Monoclonal antibody directed toward PH-20 was observed to block the binding of acrosome-reacted, but not acrosome-intact, guinea pig sperm to homologous zona pellucida in a concentration-dependent manner (Primakoff et al., 1988, Nature 335:543).

Fertilization Antigen 1 (FA-1) is a sperm specific glycoprotein that has been found in both human and mouse germ cells that was defined, originally, by the MA24 monoclonal antibody (Carron and Saling, in "Elements of Mammalian Fertilization," Volume II, Wassarman, ed., CRC Press, Boston, pp. 147-176; Naz et al., 1984, Science 225:342). The MA24 monoclonal antibody was found to recognize a 23,000 dalton monomer which may dimerize to a 46,000 dalton form and to localize at the post-acrosomal region of the sperm head and at the midpiece and principal piece of the tail (Id.). MA24 was observed to inhibit human sperm penetration of zona pellucida-free hamster eggs as well as mouse sperm penetration through mouse zona pellucida (Id.).

The M42 antigen is a high molecular weight (approximately 220,000 dalton) protein localized at the acrosomal crest of mouse sperm (Carron and Saling, in "Elements of Mammalian Fertilization," Volume II, Wassarman, ed., CRC Press, Boston, pp. 147-176; Saling and Lakoski, 1985, Biol. Reprod. 33:527). M42 monoclonal antibody appears to inhibit induction of the acrosome reaction without interfering with sperm binding to or penetration through the zona pellucida (Saling, 1986, Dev. Biol. 132:174).

PH-30 monoclonal antibody was found to bind to a target antigen localized in the post-acrosomal region of guinea pig sperm and to inhibit the fertilization of zona pellucida-free guinea pig eggs in a concentration dependent manner. PH-30 antibody appears to bind to two polypeptides having molecular weights of 60,000 and 44,000 daltons (Carron and Saling, in "Elements of Mammalian Fertilization," Volume II, Wassarman, ed., CRC Press, Boston, pp. 147-

176; Primakoff and Myles, 1983, Dev. Biol. 98:417; Primakoff et al., 1987, J. Cell. Biol. 104:141).

M29 monoclonal antibody has been found to bind to a target antigen having a molecular weight of about 40,000-60,000 daltons localized in the equatorial segment of the mouse sperm head; M29 antibody cross reacts with sperm of various species (Carron and Saling, in "Elements of Mammalian Fertilization," Volume II, Wassarman, ed., CRC Press, Boston, pp. 147-176; Saling et al., 1985, Biol. Reprod. 33:515; Saling, 1986, Dev. Biol. 117:511-519).

YWK II has been identified in human sperm by immunoaffinity chromatography using the corresponding YWK II monoclonal antibody (Carron and Saling, in "Elements of Mammalian Fertilization," Volume II, Wassarman, ed., CRC Press, Boston, pp. 147-176; Yan et al., 1987, Arch. Androl. 18:245). The YWK II antigen appears to have a molecular weight of 60,000 and/or 72,000 dalton and may be related to lactoferrin (Id.).

APz, a boar sperm plasma membrane integral protein, has been observed to be involved in the adhesion of sperm to eggs via the porcine equivalent of ZP3 (Peterson and Hunt, 1989, Gamete Res. 23:103; Peterson and Hunt, 1989, J. Cell Biol. 109:125a abstract no. 673).

Leyton and Saling (1989, Cell 57:1123-1130) studied the binding of radiolabeled murine zona pellucida or purified ZP3 to blots of separated sperm proteins and identified two zona pellucida binding proteins having molecular weights of 95,000 and 42,000 daltons. The 95,000 dalton protein that bound to ZP3 was also observed to react with an anti-phosphotyrosine antibody. It was suggested that the 95,000 dalton protein may be aggregated on the sperm

surface by ZP3, thereby stimulating tyrosine kinase activity and causing acrosomal exocytosis.

Other reports relating to molecules that have been suggested as candidates for sperm ZP3-binding protein include Macek and Shur (1988, Gamete Res. 20:93-109), relating to mouse sperm galactosyl transferase having a molecular weight of about 57,000-60,000 daltons; Ram et al. (1989, Gamete Res. 22:321-332); Huang et al., (1982, Gamete Res. 5:355-361); Topfer-Petersen et al. (1985, Histochemistry 83:139-145); Abdullah and Kierszenbaum (1989, J. Cell Biol. 108:367-375), relating to rat sperm galactose-binding protein having a molecular weight of about 49,000-54,000 daltons; Tulsiani et al. (1989, J. Cell Biol. 109:1257-1267); Jones et al. (1988, Development 102:781-792) relating to boar sperm proacrosin having a molecular weight of 53,000; Saling (1981, Proc. Natl. Acad. Sci. U.S.A. 78:6231-6235); Benau and Storey (1987, Biol. Reprod. 36:282-292); O'Rand et al. (1985, J. Exp. Zool. 235:423-428); Peterson et al. (1985, Gamete Res. 12:91-100); Sullivan and Bleau (1985, Gamete Res. 12:101-116); and Brown and Jones (1987, Development 99:333-339). However, prior to the present invention, the ZP3-binding protein had not been conclusively identified.

2.3. IMMUNOCONTRACEPTION

The immune systems of both males and females recognize sperm as foreign (Carron and Saling, in "Elements of Mammalian Fertilization," Volume II, Wassarman, ed., CRC Press, Boston, pp. 147-176). Therefore, sperm components that participate in the fertilization process may serve as immunologic targets in methods of contraception (Naz, 1990, Curr. Opinion Immunol. 2:748-751; Primakoff et al., 1988, Nature

335:543-546; Isojima, 1990, Curr. Opinion Immunol. 2:752-756; Naz and Menge, 1990, Human Reproduction 5:511-518; Talwar and Raghupathy, 1989, Vaccine 7:97-101; Griffin, 1991, Human Reproduction 6:166-172; Isojima et al., 1986, Am. J. Reproductive Immunol. Microbiol. 10:90-92). Such methods would prove superior to current contraceptives in that they would not involve administration of hormones and would therefore avoid the side effects of hormone therapy.

An antibody-mediated immune response to sperm could interfere with fertilization at any one of a number of sites because the immune system potentially gains access to sperm in a variety of locations in both the male and female genital tracts (Id.). For example, sperm could be eliminated from the functional population via nonspecific mechanisms such as agglutination or immobilization (Id.). Alternatively, a specific cellular event that is part of the fertilization process could be prevented (Id.).

While whole sperm and sperm extracts have been shown to be effective in preventing conception in experimental animal models, such preparations could not be used on a practical level for a variety of reasons (Id. and Naz, 1988, Am. J. Reprod. Immunol. 166:21). A vaccine representing one or a few sperm proteins would be more desirable; however, despite the identification of a number of sperm antigens (supra), few have been demonstrated to be targets of neutralizing antibodies in vivo and have been purified to an extent that would allow active immunization studies (Carron and Saling, "Elements of Mammalian Fertilization", Volume II, Wassarman, ed., CRC Press, Boston, pp. 147-176). Guinea pig antigen PH-20, FA-1 antigen of mouse and human germ cells, and LDH-C4, a sperm specific mitochondrial antigen found on the

sperm surface, represent three antigens that are known targets of neutralizing antibodies as demonstrated by inhibition of fertilization in vitro and induction of infertility in active immunization protocols (Id. and Goldberg et al., 1981, in "Human Reproduction", Semm and Mettler, eds., Elsevier-North Holland, Amsterdam, p. 360; Shelton et al., 1983, J. Reprod. Immunol. Suppl. 7:26; Shelton and Goldberg, 1986, Biol. Reprod. 35:873).

2.4. THE T-COMPLEX AND TESTIS-SPECIFIC GENES

The t complex, a cluster of genes located on the proximal third of mouse chromosome 17, is associated with embryonic development and fertility, and expresses phenotypes that are caused either by single gene mutations or through complex interactions of multiple loci. For example, homozygosity for a single t-lethal gene results in the arrest of the developing embryo at a specific stage, while mutations in multiple loci cause transmission ratio distortion in heterozygous t haplotype-bearing males(t/+) or sterility in males that are doubly heterozygous for t haplotypes (t^x/t^y) (Klein, 1986, in "Natural History of the Major Histocompatibility Complex," John Wiley & Sons, New York; Silver, 1985, Ann. Rev. Genet. 19:179-208, and see infra).

Spermatogonia, the stem cells that form sperm, give rise to undifferentiated spermatogenic cells called spermatocytes. Each spermatocyte undergoes two meiotic divisions to generate four haploid sperms. The haploid sperms then differentiate into mature sperm. This entire process, called spermatogenesis, takes place in the seminiferous tubules of the testis. A number of genes associated with the t-complex or that otherwise map to chromosome 17 have been shown to exert significant effects on murine spermatogenesis.

They are categorized here according to the phenotypes they exhibit.

First, a set of t-haplotype genes have been found to function in concert to cause t-bearing sperm to inactivate their wild type meiotic partners (Seitz and Bennett, 1985, Nature 313:143-144; Silver and Olds-Clarke, 1984, Dev. Biol. 105:250-252). As a result, a male heterozygous for a t-haplotype will pass the t-chromosome to over ninety percent of its progeny. This phenomenon is termed transmission ratio distortion (TRD). TRD is not the result of embryonic lethality, since female mice of the same genotype produce progeny in the classical Mendelian distribution.

The second kind of genes which affect spermatogenesis are seen in male mice carrying two complete t haplotypes. Lyon found that t-haplotypes carried at least two t-complex sterility genes (Tcs^1) (Lyon, 1986. Cell 44:357-363). Homozygosity for either one appeared to impair male fertility.

The third type of gene affecting spermatogenesis was discovered through interspecies hybrids, i.e., by breeding certain strains of Mus musculus and Mus domesticus, Mus laboratorius (mixed strains between Mus musculus and Mus domesticus) and Mus spretus, and t-haplotype-bearing Mus laboratorius and Mus spretus. These loci are called hybrid sterility (Hst) loci, because they result in sterility in the F1 male of these crosses. To date, at least two Hst genes are mapped in the t complex (Forejt and Ivanyi, 1975. Genet. Res. 24:189-206). This class of genes, like those genes involved in TRD, manifest their effects only when the animal is either heterozygous for a locus or homozygous for that locus in the context of a different genetic background.

The last two types of genes expressed during spermatogenesis are quaking (qk) (Bennett et al., 1971, Biol. Reprod. 5:30-58), which is a simple recessive unifactorial mutation, and phosphoglycerate kinase 2 (Pgk-2) (Kramer and Erickson, 1981, Dev. Biol. 87:37-45), which is expressed in the stage of spermatogenesis known as the round sperm. These genes do not seem to participate in processes or pathways in which the first three classes of genes are involved.

In order to further investigate the role of the proximal region of mouse chromosome 17 in male germ cell differentiation, Sarvetnick et al. (1989, Immunogenetics 30:34-41) set out to clone and characterize genes from this genomic region that are expressed in a testis-specific manner. Briefly, a mouse testis cDNA library of approximately 20,000 clones was screened with cDNA clones complementary to poly(A)+ RNA isolated from either mouse testis or liver, and a set of 16 cDNA clones that hybridized selectively to testis probe were identified. Northern blot hybridization confirmed the testis-specific expression of transcripts corresponding to 15 of these clones. Southern blot analysis using DNA prepared from a somatic cell hybrid featuring mouse chromosome 17 on a hamster background indicated that only one clone, named pNS2, mapped to chromosome 17. pNS2 was found to contain a portion of a larger gene, D17Si11. Sequence analysis of this gene demonstrated (i) complementary sets of alternating purine and pyrimidine residues within the corresponding RNA transcript that could form double-stranded, hairpin-like secondary structures; and (ii) a hypothetical long open reading frame complementary to the testis transcripts that appeared to be three times the size of the longest potential open reading frame present in

the transcript itself. The testis transcript, based on sequence analysis, was estimated to encode a polypeptide that was at most 187 amino acid residues in length (corresponding to a molecular weight of about 21,000 daltons). It was later found (Sarvetnick et al., 1990, Immunogenetics 31:283-284) that the transcript in fact contained an open reading frame encoding 506 amino acids (corresponding to a molecular weight of 57,000); the underestimate in the earlier publication was due to sequencing errors.

Bibbins et al. (1989, Genomics 5:139-143) reports the cloning of the human homolog of the mouse D17Sill gene. Somatic cell hybrid analysis indicated that the human gene was located on the short arm of chromosome 6 (p11-p21.1).

3. SUMMARY OF THE INVENTION

The present invention relates to a protein, termed t-complex associated testes expressed-1 (tcte-1), that is found on the surface of mature sperm and is responsible for the binding of sperm to egg during mammalian fertilization. It is based, at least in part, on the cloning and characterization of human and mouse cDNAs that encode tcte-1 protein.

The present invention provides for substantially purified nucleic acid sequences encoding tcte-1, including human TCTE-1 sequence. Such nucleic acid sequences may be linked to a promoter sequence or to at least a portion of a second gene and may be comprised in a nucleic acid vector or may exist as a transgene in a transgenic animal.

The present invention further provides for substantially purified tcte-1 protein, including human TCTE-1 protein, as well as for immunogenic fragments and derivatives thereof.

In additional embodiments, the present invention provides for vaccines comprising a tcte-1 protein, or an immunogenic fragment or derivative thereof. Alternatively, the present invention provides for vaccines comprising a nonpathogenic virus which carries a gene that encodes a tcte-1 protein or an immunogenic fragment thereof.

The present invention further provides for a method of contraception comprising administering to a subject in need of such treatment an immunogenic formulation comprising (i) a tcte-1 protein or a fragment or derivative thereof or (ii) a virus that carries a gene encoding tcte-1 or an immunogenic fragment thereof.

The present invention also provides for antibodies, including monoclonal antibodies, directed toward tcte-1, and for methods of contraception comprising administering an effective amount of such antibodies to a subject.

The present invention further provides for methods of identifying and/or diagnosing infertility that comprise detecting an abnormality in the expression and/or function of a tcte-1 protein, and for kits that may be used in such methods.

In additional embodiments, the present invention provides for methods of treating infertility that comprise correcting an abnormality in the expression and/or function of a tcte-1 protein.

3.1. NOMENCLATURE

tcte-1	t-complex associated testes expressed-1, without regard to species
Tcte-1	murine t-complex associated testes expressed-1
TCTE-1	human t-complex associated testes expressed-1

4. DESCRIPTION OF THE FIGURES

FIGURE 1. Restriction maps of Tcte-1 and TCTE-1 genomic clones.

The mouse Tcte-1 locus was cloned in two overlapping cosmids from a t^{wLub1}/t^{w5} mouse cosmid library (A). The human TCTE-1 locus was cloned in two overlapping phage clones from a human genomic library (B). The filled boxes represent regions that hybridize to cDNA, but exact junctions of the introns and exons have not been determined. The locations of restriction sites observed with the enzymes EcoRl, BamHI, XhoI, SalI, ClaI, HindIII and XbaI are indicated by the symbols shown in the rectangle on the bottom. The expanded regions were further analyzed with the rare cutting restriction enzymes NruI, BssHII, SacII, and XhoI by the abbreviations shown in the square box. The direction of transcription is from left to right.

FIGURE 2. Schematic presentation of three classes of human TCTE-1 cDNA.

Human TCTE-1 cDNAs were obtained by screening a human testis library using a mouse Tcte-1 cDNA clone as a probe. Three classes of clones were obtained. Boxes filled with the same pattern indicate that different clones shared the same sequence. DNA sequences flanking the alternative regions of the TCTE-1 clones are underlined, and sequences that could be alternative polyA addition signals are boxed. The scale is indicated by a bar.

FIGURE 3. Nucleotide sequence and deduced amino acid sequence of TCTE-1 cDNA.

The nucleotide sequences of two human TCTE-1 cDNA clones, pH1 (SEQ. ID NO:1) and phTCTE-1 (SEQ. ID NO:2), are displayed from 5' to 3' direction with the number "1" denoting the 5' end of the transcript. The

phTCTE-1 sequence is shown above the pH1 sequence. Identity of sequence is indicated by a dash. Since the pH1 sequence encodes a much smaller protein than phTCTE-1, the amino acid sequence (SEQ. ID NO:3) of phTCTE-1 is shown. Numbering of nucleotide sequence is shown on the right with those of phTCTE-1 underlined. Numbering of amino acid residues is shown on the left. The palindromic sequences in the coding region and the (CA/GT) repeating motifs in the 3' untranslated region are underlined.

FIGURE 4. Comparison of human TCTE-1 and mouse Tcte-1 (Sarvetnick et al., 1990, Immunogenetics 31:283-284) nucleotide sequences by dot matrix analysis.

FIGURE 5. Homology between human TCTE-1 (SEQ. ID NO:4) and mouse Tcte-1 (SEQ. ID NO:5) protein sequences.

Human phTCTE-1 (SEQ. ID NO:2) encodes the largest open reading frame among all the isolates. The translated open reading frame is shown on the upper line. For comparison, the mouse protein sequence is shown below the human sequence. A line indicates identity, and one dot represents a conserved change.

FIGURE 6. Hydropathy profile of Tcte-1 and TCTE-1 proteins.

The Kyte-Doolittle hydropathy profiles of mouse Tcte-1 (SEQ. ID NO:5) and human TCTE-1 (SEQ. ID NO:4) are presented in A and B, respectively. Hydrophobic regions of proteins extend above the zero line, whereas hydrophilic regions extend below the line.

FIGURE 7. Duplicated amino acid motif of Tcte-1 and TCTE-1 proteins.

The polypeptide sequences from residues 337 to 436 (SEQ. ID NO:6) of the Tcte-1 gene and residues 334 to 433 (SEQ. ID NO:7) of the TCTE-1 gene are shown in

A and B, respectively. Identical residues are underlined. The duplicated motifs are boxed.

FIGURE 8. Generation of fusion proteins of TCTE-1 and Tcte-1 and antibodies recognizing the fusion proteins.

(A) Total protein lysates from bacteria carrying p77.1 plasmid (lane 1), p77.3 plasmid (lane 2), p77.4 plasmid (lane 3) and p77.2 plasmid (lane 4). M is the marker lane.

(B) The insoluble fraction of protein lysates from p77.1 bearing bacteria.

(C) Immunoprecipitation of proteins from subcellular fractions of testicular cells with antiserum raised against mouse Tcte-1 fusion protein. Mouse testicular cells (T) were isolated and labeled with [^{35}S]-methionine. Subsequently, fractionation was performed on these cells to separate nuclear(N), cytoplasmic and membrane(C), and mitochondrial fractions(M). Protein lysates were prepared and precipitated with either anti-Tcte-1 serum (right panel) or preimmune serum (left panel).

FIGURE 9. Evidence that p56 from testis is the Tcte-1 protein.

A. [^{35}S]-methionine labeled proteins from either testicular cells or <u>in vitro</u> translated products were precipitated with preimmune or affinity purified anti-Tcte-1 antibodies and subjected to SDS-PAGE analysis. Total testicular protein lysates were used in lanes 1 and 2. <u>In vitro</u> translation products of anti-sense strand Tcte-1 RNA made by <u>in vitro</u> transcription were used in lanes 3 and 4. <u>In vitro</u> translation products of the sense strand of Tcte-1 RNA were used in lanes 5 and 6. Product precipitated by purified rabbit preimmune IgG was used in lanes 1, 4

and 6. Products precipitated by affinity purified anti-Tcte-1 antibodies was used in lanes 2, 3 and 5.

B. One dimensional peptide mapping of p56 and p62 with V8 protease. Gel slices containing radiolabelled p56 and p62 precipitated from testicular cells were excised after first-dimension SDS-PAGE, mixed with E. coli-made Tcte-1 fusion protein and subjected to a second round of SDS- PAGE analysis. Proteins were digested with 200 ng V8 protease during the second round of electrophoresis. The right panel (R) shows Coomassie blue staining pattern of the gel after electrophoresis while the left panel (L) is the radiolabelled peptide pattern from the same gel. The proteins analyzed were as follows: Lane A, p62 and Tcte-1 fusion proteins; Lane B, p56 and Tcte-1 fusion proteins. Results from two different experiments are shown. Corresponding bands are joined by lines.

FIGURE 10. Tissue and subcellular distribution of Tcte-1 proteins determined by Western analysis.

Western blotting analysis was performed on protein lysates from a variety of tissues and different subcellular fractions of testicular cells and sperm of a male 129 mouse with either affinity purified anti-Tcte-1 antibodies (right panel) or preimmune rabbit IgG (left panel). B, brain; K, kidney; L, liver; S, spleen; T, total testicular cells; SP, sperm SDS soluble fraction; TM, TC and TN are abbreviations for mitochondrial, cytoplasmic and nuclear fractions of testicular cells, respectively.

FIGURE 11. Localization of Tcte-1 RNA in testis by in situ hybridization.

A Bluescript based plasmid, pj26.1, containing the PstI-EcoRI fragment of the 3' untranslated region of Tcte-1 cDNA was linearized and transcribed with T3 and T7 polymerase to generate antisense (AS) and sense

(S) probes, respectively. PS: pachytene spermatocyte, RS: round spermatocyte. In situ hybridization experiments on testis sections were performed as described in Materials and Methods.

FIGURE 12. Localization of Tcte-1 proteins on testicular sections by immunofluorescence.

Omni-fixed mouse testicular sections were first stained with 5 micrograms of preimmune rabbit IgG (control) and affinity purified rabbit anti-Tcte-1 antibodies (alpha-Tcte-1), and then treated with biotin conjugated goat antirabbit IgG. Antigen-antibody complexes were detected with FITC labelled streptavidin. Nuclei were revealed by DAPI. Photographs were taken with a Zeiss-phase contrast fluorescence microscope. 1. Phase contrast photograph of a cross section of a seminiferous tubule. 2. DAPI staining pattern of the same field. 3. FITC staining pattern of the same field.

FIGURE 13. Localization of Tcte-1 proteins on sperm by immunofluorescence.

The method used was the same as that employed in Fig. 12. 1. Phase contrast photograph of paraformaldehyde fixed sperm. 2. DAPI staining pattern of the same field. 3. FITC staining pattern of the same field.

FIGURE 14. Tcte-1 protein is present on the plasma membrane of the intact acrosome.

FIGURE 15. Analysis of Tcte-1-LT transgenic mice.

A. The scheme of Tcte-1-LT transgene construction.

A Tcte-1-LT fusion gene was constructed by cloning the 2.3 Kb HindIII-KpnI fragment of pUCT into KpnI, HindIII linearized pj59.1 plasmid containing the 5.8 Kb upstream region of mouse Tcte-1 gene. The 3' KpnI site of the resulting plasmid, pj66.1, was

converted to a BamHl site to form the plasmid pj66.2. The 8.1 Kb BamHl fragment was used to produce transgenic mice.

B. Southern analysis of four transgenic lines.

PstI digested genomic DNA from different transgenic lines were electrophoresised, blotted, and hybridized with [^{32}P]-dCTP labeled Large T antigen probes.

C. Northern blot analysis of RNA from tissues of transgenic mice.

10 micrograms of total RNA from 6 tissues were used for Northern analysis. B, brain; K, kidney; L, liver; S, spleen; T, testis; Lg, lung.

FIGURE 16. Southern blot of DNA form various species hybridized to labelled mouse Tcte-1 probe. DNA's in lanes 1-12 (from left to right) are respectively, cow, pig (lanes 2 and 3), dog, rabbit, guinea pig, human, monkey, chicken, Xenopus laevis, zebra fish, and molecular markers.

FIGURE 17. Western Blot analysis showing the binding of monoclonal antibody 4F7 to human TCTE-1 fusion protein (lane 2) but not control (protein expressed by vector lacking TCTE-1-encoding sequences.

FIGURE 18. Immunofluorescent staining of human sperm with polyclonal antisera directed toward TCTE-1.

5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) nucleic acids;
(ii) proteins and peptides;
(iii) antibodies of the invention;
(iv) vaccines;
(v) immunocontraception; and

(vi) additional utilities of the invention.

5.1. NUCLEIC ACIDS

The present invention provides for substantially purified nucleic acid molecules encoding tcte-1, including nucleic acid molecules encoding human TCTE-1.

In particular, the present invention provides for substantially purified nucleic acid molecules having either:

i) a sequence substantially as depicted in Fig. 3 for human TCTE-1 (SEQ. ID NO:2);

ii) a sequence substantially as depicted in Fig. 3 for the portion extending between about nucleotide 149 to about nucleotide 328 (SEQ. ID NO:2), encoding the N-terminal 60 amino acid residues of TCTE-1 protein;

iii) a sequence substantially as depicted in Fig. 3 for TCTE-1 (SEQ. ID NO:2) for the portion extending between about nucleotide 329 to about nucleotide 1672;

iv) a sequence encoding human TCTE-1 protein having an amino acid sequence (SEQ. ID NO:4) substantially as depicted in Fig. 5;

v) a sequence as contained in phTCTE1 (SEQ. ID NO:2) as deposited with the American Type Culture Collection having accession number _____;

vi) a sequence as contained in 1hTCTE1g as deposited with the American Type Culture Collection having accession number _____; and

vii) a sequence as contained in pmTCTE1 as deposited with the American Type Culture Collection, having accession number _____.

The nucleic acid molecules of the invention may be comprised in a nucleic acid vector including, but not limited to, a plasmid, cosmid, bacteriophage, or virus, or may be comprised in a foreign genome, as when the nucleic acid molecule of the invention is used to create a transgene in a non-human transgenic animal.

The language "substantially as depicted" should be construed to mean that the nucleic acid molecules of the invention may differ in sequence from the sequence depicted in the figures at a number of residues that do not exceed five percent of the total sequence.

It may be desirable to insert a nucleic acid molecule of the invention into an expression vector, so as to enable recombinant expression of tcte-1. Such a vector may preferably also contain a promoter element that may be used to control expression of tcte-1.

The present invention provides for substantially pure tcte-1 nucleic acid molecules linked to a promoter element, which may, or may not, be comprised in a vector. Suitable promoter elements include, but are not limited to, sperm or testis specific promoters such as phosphoglycerate kinase 2 promoter as well as the following promoters that are not sperm or testis-specific: the CMV promoter, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1440-1445), the regulatory sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the

β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phophatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94);

myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In addition to nucleic acid molecules that encode tcte-1 linked to a promoter, the present invention also provides for nucleic acid molecules that encode tcte-1 and are linked to at least a portion of another gene, which may, for example, encode a fusion protein. Such constucts may, or may not, be comprised in a vector molecule. For example, a tcte-1 gene, or a portion thereof, may be linked to a nucleic acid encoding an immunoglobulin molecule, or a portion thereof.

The present invention also provides for substantially purified nucleic acid molecules that are at least about ten base pairs in length and that (i) correspond to a portion of the nucleic acid sequence (SEQ. ID NO:2) for TCTE-1 substantially as depicted in Fig. 3 or (ii) encode a portion of the amino acid sequence (SEQ. ID NO:4) for TCTE-1 substantially as depicted in Fig. 5. Such molecules may be linked to a promoter and/or at least a portion of another gene and may be comprised in a nucleic acid vector.

The present invention further provides for a microorganism, genetically engineered cell, or transgenic animal into which has been introduced a nucleic acid molecule of the invention. The nucleic acid molecule may have been introduced into the microorganism, cell, or transgenic animal by any method known in the art, including, but not limited

to, microinjection, transfection, transduction, electroporation, etc. or it may have been inherited from a parent microorganism, cell, or transgenic animal that had received a nucleic acid molecule of the invention through such techniques.

The present invention also provides for substantially purified nucleic acid molecules corresponding to tcte-1 encoding sequences of other species that encode proteins that are at least eighty percent homologous to the c-terminal half of TCTE-1 or Tcte-1 (Sarvetnick et al., 1990, Immunogenetics 31:283-284).

5.2. PROTEINS AND PEPTIDES

The present invention provides for substantially purified tcte-1 protein and peptide molecules, as well as derivatives and functional equivalents of such molecules. All of the proteins described in this section may be referred to as tcte-1 proteins.

In particular, the present invention provides for the following:

(i) substantially purified protein having a sequence substantially as depicted in Fig. 5 for human TCTE-1 (SEQ. ID NO:4);

(ii) substantially purified protein having a sequence substantially as depicted in Fig. 5 for the portion extending between amino acids 1 and 60 for human TCTE-1 (SEQ. ID NO:4);

(iii) substantially purified protein having a sequence substantially as depicted in Fig. 5 for the portion extending between amino acids 61 and 503 for human TCTE-1 (SEQ. ID NO:4);

(iv) an immunogenic fragment of substantially purified protein having a sequence substantially as depicted in Fig. 5 for human TCTE-1 (SEQ. ID NO:4),

including a fragment of such protein that has been rendered immunogenic by chemical modification or coadministration with adjuvant.

A protein fragment, according to the invention, is at least five amino acids in length.

The present invention further provides for such proteins and protein fragments that are comprised in a larger protein molecule. For example, such proteins and protein fragments may be comprised within a fusion protein.

The present invention also provides for functional equivalents of the proteins and protein fragments as described supra. In functionally equivalent molecules, the amino acid sequence is substantially as depicted in Fig. 5 except that functionally equivalent amino acid residues are substituted for residues within the sequence, resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Also included within the scope of the invention are derivatives of such proteins or fragments thereof,

including derivatives formed by glycosylation, proteolytic cleavage, phosphorylation, linkage to an antibody molecule or other cellular ligand, etc.

The proteins and protein fragments of the invention may be prepared by any method known in the art, including purification from a natural source (including purification by polyacrylamide gel electrophoresis, immunoprecipitation or affinity chromatography), chemical synthesis, and recombinant DNA technology expression systems.

The language "substantially as depicted" should be construed to mean that the protein molecules or fragments of the invention may differ in sequence from the sequences depicted in Figure 5 by a number of non-functionally equivalent residues that do not exceed five percent of the total sequence. Any number of functionally equivalent residues may be substituted but these, preferably, do not account for more than twenty percent of the total sequence.

The present invention further provides for a substantially purified protein having a homology of at least eighty percent to a corresponding length of tcte-1 sequence, which is substantially identical to a protein found in sperm lysates. In preferred embodiments, this protein has a molecular weight of about 50kD.

The present invention also provides for a substantially purified protein having a region of homology of at least 20 amino acids that are at least seventy-five percent homologous to a corresponding length of tcte-1 sequence, which is substantially identical to a protein found in liver and brain extracts. In preferred embodiments, this protein has a molecular weight of about 35 kD.

The present invention further provides for tcte-1 proteins of other species that are at least eighty percent homologous to TCTE-1 or Tcte-1 in the c-terminal half of the molecule.

The present invention further provides for fusion proteins that comprise all or a portion of a tcte-1 protein, such as human TCTE-1 protein. For example, but not by way of limitation, the present invention provides for fusion proteins that comprise all or a portion of TCTE-1 as well as all or a portion of an immunoglobulin protein. In another non-limiting embodiment of the invention, a fusion protein may be constructed in which a BamH1 fragment containing 430 amino acids of TCTE-1 protein may be ligated into a suitable expression vector.

5.3. ANTIBODIES OF THE INVENTION

According to the invention, the tcte-1 proteins, protein fragments, and derivatives of the invention may be used as immunogens to generate antibodies which may be polyclonal or monoclonal.

To further improve the likelihood of producing an immune response, the amino acid sequence of a protein of the invention may be analyzed in order to identify portions of the molecule which may be associated with increased immunogenicity. For example, the amino acid sequence may be subjected to computer analysis to identify surface epitopes, as illustrated in Fig. 6, which presents Kyte-Doolittle hydropathy profiles of mouse Tcte-1 and human TCTE-1. Hydrophilic regions are more likely to be exposed to the cell surface than are hydrophobic regions, and hence are more likely to be immunogenic.

Alternatively, the deduced amino acid sequences of tcte-1 from different species could be compared,

and relatively non-homologous regions be identified. These non-homologous regions would be more likely to be immunogenic across various species. For example, the amino terminal 60 amino acid residues of mouse and human tcte-1 appear to have divergent sequences. These portions of tcte-1 may prove to be immunogenic across species.

For preparation of monoclonal antibodies, any technique which provides for the production of antibody molecules may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256: 495-497), the trioma technique, the human B-cell hydridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc., pp. 77-96) and the like are within the scope of the present invention.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1983, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of tcte-1. For the production of antibody, various host animals can be immunized by injection

with tcte-1 protein, or fragment or derivative thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, MPL + TDM + CWS (RIBI Biochem.) and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and, Corynebacterium parvum.

A molecular clone of an antibody to a tcte-1 epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the 2 Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

As described in Section 6.2.3, infra, rabbit polyclonal antiserum to a Tcte-1 fusion protein was produced.

As described in Section 8, infra, monoclonal antibody 4F7, directed toward TCTE-1, was produced. Accordingly, the present invention provides for monoclonal antibody 4F7 as well as any other antibody that is capable of competing with monoclonal antibody 4F7 for binding to its target epitope.

5.4. VACCINES

The present invention provides for vaccines comprising either (i) a tcte-1 protein or a fragment thereof or (ii) a nonpathogenic virus which carries a gene that encodes a tcte-1 protein or an immunogenic fragment thereof.

According to the invention, a vaccine comprising a tcte-1 protein or a fragment or derivative thereof as set forth in Section 5.2, supra, may further comprise an adjuvant, such as, but not limited to, Freund's adjuvant or Bacille Calmette-Guerin (BCG), and may also comprise a suitable pharmaceutical carrier, including but not limited to saline, dextrose or other aqueous solutions. An effective amount should be administered, in which "effective amount" is defined as an amount of tcte-1 protein or a fragment or derivative thereof that is capable of producing an immune response in a subject. The amount needed will vary depending upon the antigenicity of the tcte-1 protein, fragment, or derivative used, and the species and weight of the subject to be vaccinated, but may be ascertained using standard techniques. In preferred, non-limiting, embodiments of the invention, an effective amount of vaccine may produce an elevation of anti-tcte 1 antibody titer to at least three times

the antibody titer prior to vaccination. In a preferred, specific, nonlimiting embodiment of the invention, approximately 50μg to 1mg and preferably 50μg to 200 mg of TCTE-1 may be administered to a human subject.

The present invention further provides for vaccines comprising a nonpathogenic virus which carries a gene that encodes a tcte-1 protein or an immunogenic fragment thereof, in which the virus may be either a live virus or may be inactivated. The virus is nonpathogenic in that it does not exert a sustained deleterious effect on a vaccinated subject, although it may produce mild, transient discomfort shortly after vaccination.

Suitable viruses which may be utilized include, but are not limited to, vaccinia, adeno associated virus, retrovirus, and other DNA viruses.

The gene that encodes a tcte-1 protein or an immunogenic fragment thereof may encode the tcte-1 protein or fragment exclusively or may encode tcte-1 protein or fragment comprised in a fusion protein. For example, it may be desirable to engineer the tcte-1 protein or fragment such that it is expressed on the surface of the virus, e.g. as part of a viral coat protein. The gene encoding tcte-1 protein or fragment may be inserted into the viral genome using standard molecular biology technology. The gene may comprise a nucleic acid according to Section 5.1, supra.

An inactivated virus according to the invention is a virus that is incapable of replication and/or infection. For example, the virus may lack the enzymes necessary for replication, although it may still be capable of entering a cell. Alternatively, the virus may be chemically inactivated, or inactivated by a physical agent such as heat or

irradiation, so that it is rendered incapable of replication and/or infection.

5.5. IMMUNOCONTRACEPTION

In additional embodiments, the present invention provides for methods of immunocontraception. The term "immunocontraception" refers to contraception, i.e., the inhibition of fertilization, that is mediated by an immune response which may be effected by antibodies and/or cells. The extent of inhibition should be at least about fifty percent relative to untreated controls.

In particular embodiments, the present invention provides for a method of immunocontraception comprising administering to a subject in need of such treatment an effective amount of a vaccine as described in Section 5.4., supra. Such a vaccine may comprise either a tcte-1 protein or a fragment or derivative thereof or a nonpathogenic virus which carries a gene that encodes a tcte-1 protein or an immunogenic fragment thereof.

Alternatively, the present invention provides for a method of immunocontraception comprising administering to a subject in need of such treatment an effective amount of antibody directed toward tcte-1, as described in Section 5.3, supra. An effective amount of antibody is an amount that will inhibit fertilization by about fifty percent and, preferably, by about seventy-five percent.

Subjects which may be treated by the methods of the invention include any organism that produces a tcte-1 protein, such as, but not limited to, humans, non-human primates, dogs, cats, rodents, livestock, etc. Either sex may be treated, but it is preferable that females be used as subjects because infertility

produced by the invention may be circumvented (for example, by in vitro fertilization or antibody titration) more easily in females compared to males.

Vaccine or antibody may be administered locally or systemically by any method known in the art, including, but not limited to, intravenous, subcutaneous, intramuscular, intravaginal, or oral routes.

Vaccine or antibody may be administered in a suitable, nontoxic pharmaceutical carrier, may be comprised in microcapsules, and/or may be comprised in a sustained release implant.

Vaccine may desirably be administered at serial intervals in order to sustain antibody levels.

Vaccine or antibody of the invention may be used in conjunction with other contraceptive methods.

As exemplified in Section 6.2.6., anti-tcte-1 antibody was observed to inhibit the binding of sperm to egg.

5.6. ADDITIONAL UTILITIES OF THE INVENTION

The present invention may also be used in methods for (i) selecting sperm of a particular genotype; (ii) identifying and/or detecting infertility; (iii) improving fertility; and (iv) producing interspecies hybrids.

In particular embodiments, the present invention provides for methods for selecting sperm of a particular genotype comprising (i) producing a transgenic non-human animal of one species that carries a transgene encoding a tcte-1 protein with endogenous or an alternative promoter derived from a second species of animal in which the transgene is located on the same chromosome as a gene of interest, such that a diploid cell of the transgenic animal

contains only one copy of the chromosome that carries the transgene and the gene of interest in its karyotype (i.e., is heterozygous for the gene of interest and for the tcte-1 transgene);
(ii) collecting sperm from the transgenic animal;
(iii) exposing the sperm to protein that binds to tcte-1 protein and is bound to a support that renders the protein retrievable, under conditions that promote the binding of tcte-1 to the protein; (iv) retrieving the protein bound to sperm via tcte-1; and
(v) releasing the sperm from the protein.

The protein may be any protein that specifically binds to tcte-1, such as, for example, and not by way of limitation, an antibody or an oocyte protein.

The protein may be bound to a support, including, but not limited to, an egg (as in the case of natural oocyte protein), plastic, glass, magnetic beads, resin, latex, etc. and may be bound via a molecule bound to the protein.

The protein may be retrieved by collecting the support. For example, protein bound to an a piece of plastic or glass may be exposed to sperm that express tcte-1 (tcte-1(+)) or do not express tcte-1 (tcte-1(-)) under conditions that permit binding of tcte-1 to protein. Tcte-1(-) sperm may then be washed away, leaving behind from tcte-1(+) sperm that are bound to protein on the plastic or glass. Similarly, if protein is bound to magnetic beads, the beads may be exposed to tcte-1(+) and tcte-1(-) sperm under conditions that permit binding of tcte-1 to protein, and then the beads, which have bound to tcte-1(+) sperm, may be collected by a magnet. Similar embodiments may be deduced by one skilled in the art.

Conditions that promote the binding of tcte-1 to protein include, but are not limited to, conditions

that are similar to those encountered in vivo, or those typically used for antibody antigen binding.

Sperm may be released from the protein by standard methods, e.g. those used to release proteins from immuno-affinity columns.

Because, as result of meiosis, the sperm of the transgenic animal consists of two populations, only one of which carries the chromosome bearing the tcte-1 transgene and the gene of interest, this method may be used to select sperm that carry the gene of interest. The selected sperm may then be used to fertilize an oocyte that may develop into an animal that carries the gene of interest.

For example, but not by way of limitation, it may be desirable to produce animals of a particular sex. Accordingly, the transgene may be inserted into the X or Y chromosome of a transgenic animal. The foregoing method may then be used to select sperm that carries the X or the Y chromosome.

The transgene may be inserted into a particular chromosome by any method known in the art. For example, a number of transgenic animals may be produced, and then animals that carry the transgene on the desired chromosome may be selected, e.g. by in situ hybridization of nucleotide probes to chromosomes, by somatic cell hybrid analysis, or by classical genetics.

In another example, but not by way of limitation, the methods of the invention may be used to improve the efficiency of inheritance of a gene of interest that is a transgene. It may be desirable to express the product of gene "A" in a transgenic animal, and to produce offspring of that transgenic animal that also express the product of gene "A". A difficulty has been that a transgenic animal heterozygous for gene

"A" may only pass gene "A" to half of its offspring. According to the invention, by "tagging" gene A with tcte-1, sperm carrying gene "A" may be selected and used to produce embryos, thus ensuring that virtually all of the animal's offspring may carry gene "A".

The gene of interest may be tagged by inserting tcte-1 on the same chromosome that bears gene "A", as outlined above. Alternatively, gene A may be linked to a gene encoding tcte-1 and then used to produce a transgenic animal that carries both genes in tandem.

Transgenic animals may be produced by any method known in the art, including but not limited to microinjection, e.g. according to the method set forth in U.S. Patent No. 4,873,191 by Wagner and Hoppe, issued October 10, 1989, transfection, transduction, electroporation, embryonic stem cells, etc. Any non-human transgenic animal species may be utilized, including but not limited to rodents, pigs, cows, horses, goats, sheep, non-human primates, etc.

Virtually any gene of interest may be utilized, including, but not limited to, globin, factor VIII, growth hormone, insulin, LDL receptor, etc.

In additional embodiments, the present invention provides for methods of identifying and/or diagnosing infertility in males or females. For example, the condition of infertility may be identified in a subject and/or the cause of infertility may be diagnosed. A subject may be a human or a non-human subject.

In one specific, non-limiting embodiment, the present invention provides for a method of identifying and/or diagnosing infertility in a test male subject comprising detecting the presence of a tcte-1 protein on the surface of sperm obtained from the subject, quantifying the amount of tcte-1 protein (by standard

techniques including, but not limited to, immunofluorescence, flow sorting, histochemistry, Western blot, etc.) either in terms of presence or absence of the protein on a single sperm or in a population of sperms or in terms of the absolute amount of tcte-1 expressed by a single sperm or in a population of sperms, and then comparing the amount of tcte-1 quantified with the amount of tcte-1 protein expressed in a comparable sample of sperm from a normal male subject, in which an aberrancy in the test subject relative to the normal subject may indicate that the test subject is infertile, and may positively correlate with infertility. Because tcte-1 is present on acrosome intact but not acrosome-reacted sperm, an aberrancy in tcte-1 expression may reflect on abnormality in the acrosome or acrosome reaction in the test subject.

Tcte-1 may be detected using, for example, tcte-1 ligand, including polyclonal or monoclonal antibody or the egg protein that binds to tcte-1; binding of ligand may be detected by directly or indirectly (e.g. via secondary antibody) labeling the ligand.

For example, if a tcte-1 protein is not expressed on the surface of any sperms obtained from the test subject, this indicates that the test subject is infertile.

Also, with regard to quantification of a tcte-1 protein by its presence or absence on individual sperms, the absence of a tcte-1 protein on greater than 30 percent, preferably greater than 50 percent, and most preferably greater than 75 percent of sperms supports a diagnosis of infertility. Similarly, the absence of tcte-1 protein on greater than 30 percent, preferably greater than 50 percent, and most

preferably greater than 75 percent of sperms of a subject may identify the subject as being infertile.

In further related embodiments of the invention, where quantification of tcte-1 protein is performed by measuring the amount of tcte-1 protein in a population of sperms, an amount of tcte-1 protein that is less than 70 percent, preferably less than 50 percent, and most preferably less than 25 percent of the amount found in a normal subject supports a diagnosis of infertility and may identify the test subject as being infertile.

In additional embodiments, the present invention provides for methods of identifying abnormal function of tcte-1 protein of a test subject comprising measuring the ability of tcte-1 of the test subject (either bound to sperm or otherwise) to compete with tcte-1 from a normal subject for binding to a ligand. Suitable ligands include the natural ligand for tcte-1, as is expressed by the oocyte, as well as antibody, monoclonal or polyclonal, directed toward tcte-1. In a preferred embodiment, the ligand is monoclonal antibody 4F7. For example, the ability of "x" amount of unlabeled tcte-1 from a test subject to bind to ligand may be measured in the presence or absence of labeled tcte-1 from a "y" amount of normal subject, and then compared with the ability of "x" amount of unlabeled tcte-1 from a normal subject to bind to ligand in the presence or absence of "y" amount of labeled tcte-1 from a normal subject. If tcte-1 from the test subject competes with labeled tcte-1 differently from tcte-1 from the normal subject, particularly if tcte-1 from the test subject competes poorly for ligand binding, this supports a diagnosis of infertility and may identify the test subject as being infertile.

In still further embodiments, the ability of tcte-1 protein to bind to natural ligand from a female test subject may be used to diagnose and/or identify infertility in the female subject. For example, if ligand from the female test subject competes inefficiently with ligand from a normal subject for tcte-1 binding, this would support a diagnosis of infertility and may indicate that the subject be identified as infertile.

The present invention further provides for a diagnostic kit that may be used to diagnose and/or identify male infertility. The kit may preferably comprise polyclonal or monoclonal primary anti-tcte-1 antibody together with detectably labeled secondary antibody. For example, the secondary antibody may be fluorescently labeled, or may be labeled with an enzyme, such as horseradish peroxidase. The kit may further comprise normal tcte-1, to be used as a standard of reference and/or a positive control.

The ability of primary anti-TCTE-1 polyclonal antibody, bound to fluorescently labeled secondary antibody to detectably label tcte-1 on some, but not all, sperms obtained from a test subject is illustrated in Figure 18.

The present invention also provides for methods for improving fertility comprising augmenting the amount of tcte-1 expressed by sperm. An increased amount of tcte-1 expressed at the sperm surface may result in increased adhesion of sperm to egg. In order to augment the level of tcte-1, a nonhuman transgenic animal may be produced that carries, as a transgene, a gene encoding tcte-1 that (i) is under the control of a relatively strong promoter (which may be an inducible promoter); (ii) is engineered to encode a tcte-1 that has an increased binding affinity

for the egg; and/or (iii) is present in multiple copies.

In further embodiments, the present invention provides for a method of improving fertility comprising selecting, from a population of sperm in which certain sperm are deficient in tcte-1, those sperm that express tcte-1. For example, tcte-1-bearing sperm may be selected via directly or indirectly fluorescently labeled anti-tcte-1 antibody followed by fluorescence activated cell sorting. The population of sperm enriched for tcte-1 expression may then be used for in vitro fertilization or artificial insemination.

In still further embodiments, sperm deficient in normal tcte-1 may be artificially coupled to normal tcte-1, thereby rendering the sperm capable of egg binding. For example, tcte-1 protein (or a portion thereof that participates in egg binding), may be coupled to an anti-sperm antibody, for example, as part of a fusion protein, or via other chemical coupling (covalent or noncovalent). Particularly if sperm/egg binding is to be accomplished in vitro, the anti-sperm antibody may be directed toward any antigen on the sperm surface, including tissue specific as well as tissue non-specific antigens (e.g. PH-20, PH-30, acrosin, SP10, etc.). The association of normal tcte-1 protein with sperm achieved via the anti-sperm antibody may render the sperm capable of fertilization, thereby improving fertility.

The present invention further provides for methods for producing an interspecies hybrid comprising mating a non-human animal of a first species to a non-human transgenic animal of a second species which carries, as a transgene, a gene that encodes a protein having the characteristics of tcte-1

protein derived from the first species which is expressed by the sperm of the transgenic animal.

6. EXAMPLE: CLONING AND CHARACTERIZATION OF TCTE-1

6.1. MATERIALS AND METHODS

6.1.1. STANDARD MOLECULAR BIOLOGY TECHNIQUES

Cloning and library screening were performed according to standard procedures as described in Maniatis et al., 1982, in "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. Enzymatic reactions involved in recombinant DNA technology were performed using commercially available enzymes with conditions recommended by manufacturer(New England Biolab.). Alkaline transfer onto nylon membranes (Genescreen®) was employed in Southern transfer experiments. Hybridization procedures were performed according to the method developed by Church and Gilbert (1984, Proc. Natl. Acad. Sci. U.S.A. 81:1991-1995). Random primer method (Feinberg and Vogelstein, 1984, Anal. Biochem 137:266-267) was used to generate ^{32}P labelled probes. DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467) using a T7 Sequenase kit (from United States Biochemical).

6.1.2. COSMID MAPPING

A mouse cosmid library, produced from a t^{w5}/t^{hbl} double heterozygote mouse, was provided by John Schimenti.

Restriction maps of each cosmid clone were generated according to a partial digestion/ indirect-end-labeling method described by Schimenti et al. (1987, J. Mol. Biol. 194:583-594). Briefly, DNA from each cosmid clone was digested with either SalI or ClaI, which recognizes a single site within the vector DNA and relatively few sites within the inserted mouse DNA. This allows all of the cosmid DNAs to be linearized at a defined site within the vector sequence. Subsequently, the completely digested DNA was subjected to partial digestion with a more frequently cutting enzyme. The DNA sample was size-fractionated on a 0.5%(w/v) TBE (90mM Tris base, 90mM Boric acid and 2mM EDTA pH 8.3) agarose gel and blotted sequentially to two nylon membranes. The blotted DNAs were probed with fragments from isolated vector DNA that represent the two arms of the linearized clone. The sizes of bands that were resolved on each autoradiograph correspond to the distance between one end of the cosmid clone and sites of the restriction enzyme being used. The maps generated by this method were confirmed by electrophoretical analysis of complete digests of each clone with individual restriction enzymes or a combination of enzymes.

6.1.3. ISOLATION AND LABELING OF THE SPERMATOGENIC CELLS

Testicular cells were isolated and labelled in culture with [^{35}S]methionine as described in Silver et al. (1979, Cell 17:275-284). In brief, 2 to 6 testes were pooled in a 50ml tube containing 25 ml of 1mg/ml

collagenase solution and incubated for 10 to 20 minutes at 33°C until the seminiferous tubules were dissociated. The tubules were then washed twice with medium and resuspended in 25 ml of trypsin/DNAasel solution. The tube was placed in a 33°C water bath for 5-10 minutes. The cell mixture was pipetted up and down 50 times with a cut disposable pipet tip, layered over a 10 ml cushion of 1% BSA, and centrifuged at 1000 RPM for 10 minutes at 10°C. The resulting cell pellet was resuspended in PBS, and the cell density was adjusted to 1 x 10^7 cells/ml. 200 microcuries of [^{35}S]methionine was added and the cells were incubated at 33°C/ 5% CO_2 for 6 hours. Labeled cells were washed with PBS three times and stored at -80°C.

6.1.4. IN SITU HYBRIDIZATION

Testes from adult male mice were embedded in O.C.T. compound (Miles) and frozen at -80°C. 10 mm semi-thin sections were cut, adhered onto slides pretreated with 0.1 mg/ml poly-L-lysine (Sigma), and stored at -80°C. Immediately prior to use, slides were air-dried and fixed for 1 minute in freshly prepared 4% paraformaldehyde (Sigma) in PBS at room temperature and for 5 minutes in 70% ethanol at 0°C. Slides were hybridized with [^{35}S]-UTP-labelled riboprobes according to the procedure of Sideras et al. (1988, Proc. Natl. Acad. Sci. U.S.A. 85:218-221), exposed at room temperature for 4-6 days, developed in 1/2X Dektol (Kodak), and counterstained with Mayer's hemotoxin solution (Sigma).

6.1.5. PREPARATION OF THE FUSION PROTEINS

The NaeI to EcoRI fragment from mouse cDNA pmTcte-1 was filled in with Klenow enzyme in the

presence of all four dNTPs and subsequently ligated with the BamHl-linearized and blunted fragment of the expression vector pAR 3038. The clone containing the appropriate orientation was identified by the standard procedure (Maniatis et al., 1982, supra).

To direct expression of the Tcte-1 fusion gene, the recombinant plasmids were transfected into E. coli BL21(DE3), which carries a copy of the T7 RNA polymerase gene under control of the LacUV5 promoter. The resulting strain was grown with shaking at 37°C in 100 ml TB medium supplemented with 150ug/mL ampicillin. Isopropyl beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM when the culture reached OD600 =0.5. After another hour of shaking at 37°C, bacteria were collected and resuspended in 4 ml of lysis buffer (50 mM Tris-HCl pH 8,2 mM EDTA and 20 mM NaCl). The cells were lysed overnight on ice with the addition of lysozyme to a final concentration of lmg/ml and sodium deoxycholate to a final concentration of 0.04%. The lysate was sonicated and centrifuged at 10,000 RPM for 15 minutes in a Sorvall SS-34 rotor. The pellet was washed with lysis buffer twice and resuspended in 2 ml of lysis buffer. Lysate was subjected to preparative SDS-polyacrylamide gel electrophoresis. The fusion protein was visualized by staining part of the gel with Coomassie blue and then collected by electroelution with TAE-SDS buffer.

6.1.6. IMMUNIZATION OF RABBITS

Two female New Zealand White rabbits were used for the generation of antisera against bacteria-produced Tcte-1 protein. Rabbits were injected with 100 micrograms of purified protein in physiologic saline (0.85% NaCl) emulsified in complete

Freund's adjuvant (Difco) or with MPL+TDM+CWS adjuvant (RIBI Immunochem) subcutaneously at multiple sites along the hips and shoulders. Skin was shaved prior to injection to minimize the risk of infection. The initial injection was followed by two or three similar booster injections of purified protein emulsified with incomplete adjuvant, administered at four week intervals. Subsequent booster injections were given by intravenous injection of 50 micrograms of soluble protein in saline into an ear vein. Rabbits were bled 9 to 10 days after each booster injection.

6.1.7. SUBCELLULAR FRACTIONATION AND IMMUNOPRECIPITATION

Spermatogenic cells were homogenized using a Dounce homogenizer in sterile 10 mM Tris (pH 7.5) solution, after which an equal volume of 10mM Tris, 1 M sucrose solution was added. The cell suspension was then spun at 3000 RPM for 10 minutes in an Eppendorf variable speed microfuge. The pellet was saved as a nuclear fraction. The supernatant was further spun at 14,000 RPM for 5 minutes to collect the mitochondria. The supernatant contained the cytosolic fraction. Protein lysates were made by vortexing various subcellular fractions in RIPA buffer(50mM Tris [pH8],150mM NaCl, 1% Triton-X100, 1% sodium deoxycholic acid, 0.5%SDS) with the addition of various protease inhibitors (0.2 mg/ml phenylmethylsulfonylfluoride (PMSF); 1 microgram/ml Leupeptin; 2 micrograms/ml Antipain; 10 micrograms/ml Benzamide; 1 microgram/ml Chymostatin; 1 microgram/ml Pepstatin; 1 microgram/ml Aprotinin; and 0.2 mg/ml soybean trypsin inhibitor (SBTI). Lysates were spun in the Ti50 rotor at 40,000 RPM for 1 hour to remove debris. Immunoprecipitation was performed according to the procedures described in Antibodies (Harlow and

Lane, 1988, Antibodies, A Laboratory Manual. Cold Spring Harbor Laboratory) with the following modifications. Instead of adding antibodies directly into the lysates, preloaded Staph A beads were used. The beads were prepared by incubating either 5-10 microliters of rabbit antiserum or 1 ml hybridoma supernatant with 100 microliters of a 10% suspension of Staph A beads for one hour; the unbound antibodies were then washed away with RIPA buffer. This procedure was repeated twice and the pelleted beads were resuspended in 100 microliters of borate coupling buffer consisting of 0.1M borate and 0.15 M NACl.

6.1.8. WESTERN BLOT ANALYSIS

This analysis was performed essentially according to the standard procedures described in Bumette (1981, Anal. Biochem. 112:195-203). In brief, protein lysates were separated by SDS PAGE (10%) and electrophoretically transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA) with a solution containing 20mM Tris base, 150mM glycine, 20% methanol and 1% SDS. Membranes were blocked for nonspecific binding with 3% gelatin and 5% nonfat milk in TBS (20mM Tris, pH 7.5; 0.5M NaCL), and specific proteins hybridized with 5 micrograms of affinity purified antiserum in 6 ml antibody solution(1% Tween 20, 1% gelatin, and TBS). Bound antibodies were visualized by immunoperoxidase staining using a Vectastain ABC kit (Vector Laboratory Burlingame,CA).

6.1.9. CLEVELAND DIGEST

Protein samples were resolved by SDS polyacrylamide gel electrophoresis and the bands of interest were excised and soaked in 25% buffer A(0.5 M Tris Cl, pH 6.8; 0.4 % SDS) containing 10% glycerol.

A 15 % polyacrylamide gel was cast and its wells were washed with 25% buffer A before filling them with gel running buffer. The equilibrated strips were inserted into these wells and then overlaid with 20 microliters of 2X sample buffer (0.1 M Tris, pH 6.8 containing 4% SDS, 20% glycerol, 0.08% bromophenol blue, 4% beta-mercaptoethanol). Then, 20 microliters of V8 protease (200 fold dilutions of 1mg/ml of the stock enzyme with 25% buffer A in 10% glycerol) were added above the sample buffer. The gels were run at 4V/cm.

6.1.10. AFFINITY PURIFICATION OF ANTIBODIES THAT RECOGNIZE TCTE-1 PROTEIN

Several hundred micrograms of purified Tcte-1 fusion protein was coupled to CNBR activated Sepharose 4B beads (Pharmacia) according to the recommendations of the manufacturer. 15 ml of rabbit anti-Tcte-1 serum was passed through a column containing the beads. The bound antibodies were eluted with 0.1 M glycine, pH2.5 and neutralized with 0.1M Tris-Cl, pH 8.

6.1.11. IMMUNOFLUORESCENT STAINING OF TESTICULAR SECTIONS AND ISOLATED SPERM

The Tcte-1 protein was visualized by a procedure described in Antibodies (Harlow and Lane, 1988, supra). In brief, adult B6D2 F1 mouse testis tissue was fixed with OmniFix2.0 (An-Con Genetics, Inc). Seven micrometer paraffin embedded sections were cut and stored at 4°C. Mouse epididymal sperm were fixed with 4% paraformaldehyde on poly-L-lysine coated slides. Five micrograms of affinity- purified anti-Tcte-1 antibodies or preimmune IgG was added to slides. After 4 hours of incubation at room temperature, slides were washed with PBS and the bound IgG was reacted with a biotinylated, goat anti-rabbit IgG (1:200; Bethesda Research laboratory) and

streptavidin-linked fluorescein complex(1:200; Amersham). 4', 6-diamidino-2phenylindole (DAPI) was added to a final concentration of 1 microgram/ml to reveal the nucleus. Slides were then mounted with Tris pH 7.5 buffered 90% glycerol containing 1mg/ml p-phenylenediamine to prevent photobleaching and visualized under a fluorescence microscope.

6.1.12. PRODUCTION OF TRANSGENIC MICE

Transgenic mice were produced as described in Hogan et al., 1986, in "Manipulating the mouse embryo: A laboratory manual." Cold Spring Harbor: Cold Spring Harbor Laboratory. Approximately 100-1000 copies of the Tcte-1-LT fusion construct (Fig. 14A), included in a 8.1 Kb BamHl fragment from pj66.2, were injected into single cell stage fertilized eggs. Mice were screened for the acquisition of the transgene by analysis of DNA obtained from tail biopsies. When possible, homozygote lines were established and maintained.

6.1.13. NUCLEIC ACID ANALYSIS OF TRANSGENIC MICE

Mouse tail biopsies were digested in 1% SDS, 150mM NaCl, 10 mM TrisCl(phTCTE-1.5), 100mM EDTA(pH8.0) and 0.5mg/ml proteinase K at 55°C overnight with rotation. Genomic DNA was purified by one phenol extraction followed by one phenol-chloroform extraction, then precipitated by the addition of ammonium acetate to 1.25M and an equal amount of cold ethanol. 10 micrograms of purified DNA was digested with appropriate enzyme, electrophoresed in 0.8% agarose gels, transferred, UV crosslinked to the nylon membrane (Genescreen) and hybridized with ^{32}P labelled SV40 large T antigen probe. Total RNA was isolated from various mouse tissues using guanidinium

isothiocyanate/CsCl gradient techniques (Chirgwin et al., 1979, Biochem. 18:5294-5299). 10 micrograms of total RNA was subjected to standard Northern analysis (Maniatis et al., 1982, supra), with the modification that 0.66 M formaldehyde (rather than 2.2 M) was used in the gel.

6.2. RESULTS

6.2.1. CLONING AND CHARACTERIZATION OF MOUSE AND HUMAN TCTE-1 GENOMIC DNA

Mouse Tcte-1 cDNA clone pNS2 was used to screen a cosmid library produced from a t^{w5}/t^{hub1} double heterozygote mouse. Three independent clones were obtained. Restriction sites detected with 6 enzymes (BamHl, Clal,EcoRl, HindIII, Nrul and Sall) were mapped within each cosmid clone. The results show that these three clones overlap and cover 65 Kb of DNA (Fig. 1.A). The orientation of the gene was determined by hybridizing the restriction digested cosmid DNA with different fragments of the cDNA clone. The size of the transcriptional unit appeared to be about 10 Kb. Since CpG islands are often present in the 5' end of mammalian genes and have been thought to play an important role in transcriptional regulation, these islands were searched for by looking for the recognition sequences of rare cutting restriction enzymes, which usually contain double CpGs. Sites for four such enzymes (BssHII, NruI, SacII, Xho 1) were found clustered 3 kb downstream of the 3' end of the gene (Fig. 1). Surprisingly, there are no such enzyme sites around the 5' end of this locus.

In order to learn whether this phenomenon is significant, a similar analysis was performed on the human TCTE-1 locus using human genomic clone 1hTCTE1g. A similar organization of rare cutting sites was observed around the human gene (Fig. 1.B)

demonstrating that the presence of a CpG island on the 3' end of this locus is evolutionarily conserved.

6.2.2. ISOLATION OF THE HUMAN TCTE-1 cDNA AND COMPARATIVE ANALYSIS OF HUMAN AND MOUSE TCTE-1 GENES

The screening of one million plaques plated from a human testis cDNA library, using fragments of mouse Tcte-1 cDNA (pmTCTE1) as probes, identified 6 positive clones. Further characterization of these clones categorized them into three classes. The first class consisted of one clone, pH1, which contained a 2.6 Kb insert and hybridized strongly to both the 5' and 3' ends of the mouse sequence. The second class, termed the pH7 class, contained four clones, pH2, pH5, phTCTE-1 (originally named pH7) and pH8, which were found to have a 1.6Kb insert and to hybridize very strongly with the 5' mouse probe but weakly with the 3' probe. The third class consisted of clone pH6, which contained a 1.6Kb insert and hybridized strongly with the 3' probe but weakly with the 5' probe.

All three classes of clones were completely sequenced. This analysis revealed that the transcription pattern of the human TCTE-1 locus was complex (Fig. 2) in contrast to the simple transcription pattern of the mouse Tcte-1 gene, in which only a single transcript was produced.

The differences among the three classes of clones did not appear to be caused by cloning artifacts. For example, the longest clone, pH1 (SEQ. ID NO:1), had a deletion of 450 base pairs within the open reading frame that resulted in an 150 amino acid in-frame deletion when compared to the phTCTE-1 (SEQ. ID NO:2) sequence (Fig. 3). This deletion was not present in the pH6 clone. However, the phTCTE-1 class did not contain the 1.5 Kb 3' untranslated region which was

present in both of the other classes of cDNAs. Since three independent clones of the phTCTE-1 class have been isolated, this difference most likely arose from the utilization of different polyadenylation sites. In addition, while both clones share the same 3' 1.6 Kb fragment, pH6 is not just a partial clone of pH1, since the 5' end sequence of pH6 is not present anywhere in clone pH1, but is present in the phTCTE-1 clone (Fig. 2).

The nucleotide sequence of pH1 is highly homologous to its mouse counterpart even in the 3' untranslated region as shown by dot matrix analysis (Fig. 4). Interestingly, the complimentary CA/GT motifs in the TCTE -1 gene are similar in location and orientation to its mouse homologue. As shown in Fig. 3, the CA motif is 5' to the GT motif with about 840 bps between them. Another interesting feature of the Tcte-1 gene is the existence of palindromic sequences TCCTCCACrGGAGGA and ATCCGTCGGATGCGCCGGAT from nucleotides 218 to 232 and 319 to 339, respectively, in phTCTE-1 (SEQ. ID NO:2) (Fig. 3). The second sequence is highly conserved between human and mouse.

The phTCTE-1 clone (SEQ. ID NO:2) contained the longest open reading frame, which starts at nt 149 and ends at nt 1652 (Fig. 3). The ATG codon at nts 149-151 is probably the initiating codon because it is embedded in a sequence (GCCTCCAGCAUGG) which is highly similar to the Kozak consensus for initiation of translation in higher eukaryotes (Kozak, 1987, Nucleic Acids Res. 15:8125-8148). This hypothetical open reading frame could potentially encode a 501 amino acid polypeptide, which is similar in size to its mouse counterpart.

The predicted protein sequences were compared with the sequences in the Genbank database, but no homologous sequences were found. A comparison of the predicted amino acid sequences of Tcte-1 (SEQ. ID NO:5) and TCTE-1 (SEQ. ID NO:4) reveals striking homology between these two proteins (Fig. 5). The overall sequence homology was found to be 84% with 77% identity. However, the N terminal 60 amino acids are highly diverged between these two open reading frames. Excluding this region, the similarity was as high as 90%.

The hydropathy profiles of these two sequences were plotted with the Kyte and Doolittle program (Fig. 6), and were found to be quite similar. The mouse Tcte-1 and human TCTE-1 proteins appear to be predominantly hydrophilic except for certain regions, such as amino acid residues 68-78 and 408-428 in Tcte-1, and residues 70-90, 262-288 and 403-423 in TCTE-1. It is these residues that could potentially form transmembrane helices.

There are duplicated amino acid motifs present in both genes, as shown in Fig. 7. The functional significance of these repeats is not known. Unlike the mouse sequence, there was no significant open reading frame on the complimentary strand of the human TCTE-1 transcript. The longest open reading frame obtained was 12 kd in size, which is much smaller than the 40 Kd one encoded by the reverse strand of Tcte-1.

6.2.3. CONSTRUCTION OF GENE FUSIONS FOR THE EXPRESSION OF HUMAN AND MOUSE TCTE-1 PROTEINS AND PRODUCTION OF SPECIFIC ANTISERUM

To gain further evidence that the predicted open reading frames were of the correct size gene fusions comprising human TCTE-1 or mouse Tcte-1 were prepared

and used to express the corresponding proteins in E. coli. The NaeI-EcoRI fragment that encodes 500 amino acids of mouse Tcte-1 protein was blunted and ligated into a BamHl digested, blunted pAR 3038 vector. Plasmids containing the insert in both orientations were obtained, and designated as p77.1 and p77.3. p77.1 was subsequently shown to carry the correct orientation by digesting the plasmids with BglII.

The BamHl fragment containing 430 amino acids of TCTE-1 protein was ligated into a BamHl linearized pAR3039 vector. Recombinant plasmids (p77.2 and p77.4) carrying both orientations were obtained, and p77.2 was shown to have the right orientation by an Sphl digestion. The bacteria carrying these plasmids were then grown and induced with IPTG as described in Materials and Methods. Total protein lysates were then subjected to SDS-PAGE analysis.

As shown in Fig. 8A, human and mouse tcte-1 fusion constructs were found to produce proteins of 47 and 56 KD in size, respectively. This result was consistent with the prediction based on sequence analysis.

In order to prove that the putative Tcte-1 open reading frame was utilized in mice and to further study its expression and function, rabbit antiserum was raised against the Tcte-1 fusion protein enriched in the insoluble fraction of total bacteria protein lysates (Fig. 8B). This antiserum was able to specifically recognize both human and mouse fusion proteins.

To determine whether this antiserum could detect any mouse testicular proteins, immunoprecipitation experiments were performed. Two major proteins of 56 KD and 65 KD in size were precipitated from [^{35}S]-methionine-labelled total testicular cell lysates

(Fig. 8C). Subsequently, the IgG fraction of this serum was purified using affinity chromatography. Repeating the immunoprecipitation with the affinity purified antibodies, the only specific band precipitated was the 56 KD protein.

To further demonstrate the specificity of this antibody, the 5' part of the Tcte-1 cDNA (which covers the entire open reading frame but not the 3' untranslated region) was cloned into the Bluescript vector (Stratagene). Utilizing the T7 and T3 promoter, both sense and antisense transcripts were generated and subjected to in vitro translation reaction with rabbit reticulocyte lysates. A portion of the product from the translational reaction was immunoprecipitated with anti-Tcte-1 IgG and preimmune IgG (as control). As shown in Fig. 9A, a protein of 56kd in size was precipitated with the anti-Tcte-1 IgG from the in vitro translated products of the sense transcript (lane 5). This 56 KD protein was not recognized by preimmune IgG (lane 6), and was not produced by the antisense transcript (lane 3).

In order to prove that the 56 KD protein precipitated from the total testis lysates represented authentic Tcte-1 protein, partial proteolytic analysis on both the precipitated protein and the bacterially-expressed fusion protein was carried out. The 65 KD protein was used as a negative control (Fig. 8C). The partial V8 digestion pattern of the 56 KD protein closely resembled that of the fusion protein, while the pattern of the 65 KD protein was totally different (Fig. I-9 B), supporting the conclusion that the 56 KD protein is the Tcte-I gene product.

6.2.4. TISSUE DISTRIBUTION OF TCTE-1 PROTEIN

To determine whether the 56 KD Tcte-1 protein was testis specific, Western blot analyses with affinity-purified antibodies were performed on protein extracts from mouse brain, liver, kidney, spleen, testes and sperm fractionated by SDS-PAGE. The result is shown in Fig. 10, which clearly demonstrates the presence of a 56Kd band in the testes and sperm extracts. Interestingly, a 50 Kd protein was also identified in sperm lysates, which may be a modified form of the 56 Kd protein. Surprisingly, a prominent 35 Kd protein band was present in brain, liver and kidney lanes. Since Tcte-1 RNA expression has not been detected in liver, the 35 Kd band could result from cross-reactivity with a protein bearing partial homology to Tcte-1.

6.2.5. TCTE -1 PROTEIN IS PRESENT IN MOUSE TESTIS AND SPERM

The Tcte-1 transcript was first detected in the testis of 14 day old mice (Sarvetnick et al., 1989, Immunogenetics 30:34-41), which suggested that Tcte-1 was most likely turned on in pachytene-stage spermatocytes. In situ hybridization with [^{35}S]-UTP labelled sense or antisense Tcte-1 probes was performed on frozen testicular sections to further address this question. The results demonstrate that Tcte-1 expression is indeed first observed in pachytene spermatocytes and continues to be present in haploid sperms (Fig. 11).

To localize Tcte-1 protein products, indirect immunofluorescence techniques with the affinity-purified Tcte-1 antibody were employed on total testicular sections and isolated sperm. As is shown in Fig. 12, antibody to Tcte-1 protein stained the pachytene spermatocytes and round sperms in the

testicular section. A crescent region on the sperm head, typical of the acrosome, was strongly stained (Fig. 13). The Tcte-1 staining pattern of the spermatocyte seems to colocalize with that of DAPI, which suggests Tcte-1 protein might be a nuclear protein. However, the staining is punctate and diffused, which may also represent Golgi staining.

6.2.6. TCTE-1 IS A CANDIDATE GENE FOR A ZP3-BINDING PROTEIN

The molecular weight and localization of Tcte-1 protein is similar to a recently identified 56Kd ZP3 binding protein (Bleil and Wasserman, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:5563-5567). In order to evaluate whether Tcte-1 is the ZP3 binding protein, a number of experiments were performed.

First, the location and numbers of Tcte-1 protein on sperm were evaluated by immunofluorescence. In tact, unpermeabilized sperm were exposed to anti-Tcte-1 antibody under conditions that promote antibody binding. Anti-Tcte-1 antibody bound to the sperm surface were then visualized with fluorescently labeled anti-mouse IgG antibody. As shown in Figure 13, immunofluorescence was observed in the acrosome portion of the sperm.

Second, the location and number of Tcte-1 protein was studied using gold-labeled anti-Tcte-1 antibody. As is shown in Fig. 14, anti-Tcte-1 IgG specifically stained the plasma membrane overlying the acrosome region of intact sperm, while the preimmune serum failed to do so. Based on the measurement of gold particles bound to 14 sperm, the average numbers of binding sites for anti-Tcte-1 IgG on the intact sperm was 65 particles/square micrometer, which was significantly higher than the background number of preimmune IgG(5-6 particles/square micrometer). Using

acrosome-reacted sperm, there was no dramatic difference between anti-Tcte-1 IgG and preimmune IgG; 36 and 58 particles/square micrometer, respectively. The acrosome reacted sperm appeared to be sticky, which may have contributed to the elevated levels of binding of both IgGs. Nevertheless, the specific binding of anti-Tcte IgG to acrosome-reacted sperm was completely abolished.

Second, the effect of the anti-Tcte-1 IgG on the sperm-egg interaction was determined in a competitive sperm binding assay. The preliminary result, obtained from 15 eggs used in each experiment, indicated that preincubation of sperm with anti-Tcte-1 IgG reduced the number of sperm capable of binding to an egg from 22 sperm per egg (with buffer only)to 8 sperm per egg. The extent of inhibition was similar to that of ZP3 (5 sperm per egg), but higher than that of preimmune IgG(15 sperm per egg).

6.2.7. UPSTREAM REGION OF TCTE-1 GENE FAILED TO CONFER TESTIS-SPECIFIC EXPRESSION IN TRANSGENIC MICE

In an effort to locate the regulatory elements required for testis specific expression of the Tcte-1 gene, a construct containing 5.8 Kb of upstream sequence of Tcte-1 fused to the SV40 large T antigen was used to generate transgenic mice (Fig. 15A). Four transgenic lines were established (Fig. 15B). By Northern analysis, none of them showed testis specific expression of T antigen transcripts (Fig. 15C). This result is consistent with the notion that the 3' CpG island-containing sequence might be required for the appropriate expression of the Tcte-1 gene.

6.3. DISCUSSION

6.3.1. THE OPEN READING FRAME PRESENT IN THE OPPOSITE STRAND OF THE MOUSE TCTE-1 GENE IS NOT EVOLUTIONARILY CONSERVED

Comparison of the nucleotide sequences between human TCTE-1 and mouse Tcte-1 genes have revealed that the 70 Kd open reading frame identified on the antisense strand of Tcte-1 transcript by Sarvetnick, (1986, Genetic and biochemical studies of mouse t-haplotypes. Ph.D. thesis, State University of New York, Stony Brook) was the result of several sequencing errors. The actual size of this hypothetical reading frame was reduced to 40 Kd after correcting those mistakes. On the opposite strand of the TCTE-1 gene, the corresponding open reading frame is only 12 Kd in size.

6.3.2. THE 3' UNTRANSLATED REGION OF TCTE-1, INCLUDING THE GT/CA MOTIFS, IS HIGHLY CONSERVED BETWEEN HUMAN AND MOUSE

Since the 3' untranslated region (UTR) of Tcte-1 is highly conserved between human and mice, including the presence of complementary repeating motifs, the 3' UTR may have an important function. There are $(CA/GT)_n$ repeats in this region, which, despite widespread distribution in eukaryotic genomes, are very rare in exons. Interestingly, two copies of this repeat were identified in a single exon in both the mouse and human tcte-1 genes. These repeats may be able to form Z DNA *in vitro* (Hamada and Kakunaga, 1982, Nature 298;:396-39; Nordheim and Rich, 1983, Proc. Natl. Acad. Sci. 80: 1821-1825) but not *in vivo* (Casasnovas et al., 1989, J. Mol. Biol. 208: 537-549), may play a role in genetic recombination (Pardue et al., 1987, EMBO J. 6: 1781-1789) or may enhance transcriptional

activity of genes in plasmids (Hamada et al., 1984, Mol. Cell. Biol. 4: 2622-2630).

The 3' UTR may participate in forming secondary structures that are used to achieve translational control or RNA stability. For example, translational control during spermatogenesis may be achieved in two ways. First, translation of certain genes may be prevented despite the presence of abundant transcripts. Second, all transcripts made for later use may be stored safely until spermiogenesis, the last phase of sperm differentiation, since transcription is shut off in the cells of this stage. This type of translational regulation has been well documented during gametogenesis in general (Gold et al., 1983, Develop. Biol. 98: 392-399; Kleene et al., 1984, Dev. Biol. 105: 71-79; Zakeri et al., 1988, Dev. Biol. 125: 417-422).

Braun et al (1989, Genes Dev. 3: 793-802) used transgenic mice to demonstrate that a chimeric construct consisting of the 3' untranslated region (UTR) and 51 nucleotides of the 5'UTR of the protamine gene linked to human growth hormone gene coding sequence exhibited the same delayed translational activation. On the sequence level there is no similarity between the protamine and Tcte-1 3' UTRs. The fact that other genes under similar control, such as Pgk-2, do not share any homology with the protamine 3'UTR argues strongly that the primary signal for this recognition lies in the secondary structure of the transcript rather than the primary sequence. The observation that translation and transcription of Tcte-1 protein occurred in the same cell stage indicates that its 3'UTR is not involved in preventing the translation. However, the fact that Tcte-1 protein is present in the acrosome of mature sperm

would suggest that the 3'UTR may function to prevent degradation of the Tcte-1 mRNA.

6.3.3. THE POSSIBLE FUNCTIONS OF THE 3'CPG ISLAND DNA OF TCTE-I AND TCTE-1 GENE

CpG islands have been found to be associated with the 5' flanking region of genes in vertebrates. Methylation of these islands has been suggested as the switch mechanism to turn off transcription, implying that CpG islands could play an important role in regulating transcription (Antequera et al., 1990, Cell 62: 503-514; Bird, 1986, Nature 321: 209-213). The transcriptional control elements of several testis genes have been identified using transgenic mice (Robinson et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86: 8437-8441; Steward et al., 1988, Mol. Cell. Biol. 8: 1748-1755), but these elements do not seem to carry CpG islands. However, Tcte-1 contains CpG islands in the 3' end of the gene. This could mean that either (i) 3' CpG islands do not play a role in the regulation of Tcte-1 expression; (ii) the CpG island represents the controlling elements for another gene downstream; or (iii) the island is required for the proper expression of Tcte-1.

6.3.4. HUMAN TCTE-1 GENE UNDERGOES POSTTRANSCRIPTIONAL MODIFICATIONS

Although the sequences of mouse Tcte-1 and human TCTE-1 are quite similar, the human transcripts appear to be more heterogeneous than the mouse transcripts. Northern analysis of both human and mouse polyadenylated RNA revealed a single 2.9 Kb band in mouse RNA whereas there were many bands of different sizes in human RNA. cDNA cloning of human TCTE-1 also

demonstrated that there are at least three classes of transcripts from this locus.

Detailed analysis of the differences between the pH1 and phTCTE-1 clones revealed that transcription appears to start from multiple sites and that the 5' 56 nucleotides of phTCTE-1 are likely to correspond to the intron sequence of the pH1 transcript because the sequence from nucleotide 43 to 56 of phTCTE-1 (see SEQ. ID NO:2) (CCTCTACACCCCAG) is very similar to the consensus splice acceptor site. The cause of the internal deletion from the pH1 clone is unlikely to be the result of alternative splicing since the sequence around the corresponding region of phTCTE-1 (see SEQ. ID NO:2) (CAACCACATCGGGCTG) does not bear any resemblance to the acceptor consensus sequence. Clone pH6 represents a truncated transcript that could be the consequence of cDNA cloning. However, phTCTE-1 apparently utilizes a different poly(A) addition signal from the other two classes, which results in the deletion of most of the 3' UTR.

Since three independent clones having these characteristics were obtained, it is not likely that they represent a cloning artifact. Many genes expressed in testis undergo similar post transcriptional modification (Ben-Neriah et al., 1986, Cell 44: 577-586; Cebra-Thomas et al., 1991, Nature 349: 239-241). This, in turn, would suggest the existence of novel germ cell-specific splicing machinery and poly(A) addition factors. The presence of the class phTCTE-1 transcript in human testicular cells might be problematic if the 3' UTR is crucial for the proper translation of Tcte-1 gene as discussed supra.

6.3.5. TCTE-1 PROTEIN IS LIKELY TO FUNCTION IN THE PROCESS OF SPERMATOGENESIS AND FERTILIZATION

The finding that Tcte-1 protein is present during spermatogenesis and is localized on the surface of the acrosome indicates that Tcte-1 may function in germ cell differentiation and fertilization. Several sperm surface proteins have been identified by either immunological or biochemical criteria including a mouse M42 antigen (200/220 Kd) (Saling and Lakoski, 1985, Biol. Reprod. 33: 527-536), a mouse phosphotyrosine-containing protein (95 Kd) (Leyton and Saling, 1989, Cell 57: 1123-1130), a mouse sperm surface galactosyl transferase (60 Kd),a mouse ZP3 binding protein (56Kd) (Bleil and Wasserman, 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 5563-5567), a rat galactose receptor (54 Kd) (Abdullah and Kierszenbaum, 1989, J. Cell Biol. 108: 367-375), and a guinea pig PH20 protein (64 Kd) (Primakoff et al., 1985, J. Cell. Biol. 101: 2239-2244). It is unlikely that Tcte-1 is related to M42 or the 95 Kd protein because of the considerable difference in sizes. The sequences of Tcte-1 are entirely different from those of the galactosyl transferase gene (Sharper et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 791-795) or PH20 (Lathrop et al., 1990, J. Cell. Biol. 111: 2939-2949). The rat 54 Kd galactose receptor is not present exclusively in sperm, which is in contrast to the 56 Kd protein encoded by Tcte-1.

Three lines of evidence suggest that Tcte-1 is a candidate gene for the mouse 56 Kd ZP3 binding protein. First, their molecular weights are similar. Second, the anti-Tcte-1 antibody reacts similarly to ZP3 in both quantitative and qualitative assays from the immunogold staining data; that is, the numbers of the binding sites for ZP3 and Tcte-1 antibody per

sperm are similar and they both stained the plasma membrane of the acrosome on the intact sperm but not the acrosome reacted sperm. Third, both anti-Tcte-1 IgG and ZP3 inhibited the binding of sperm to egg in vitro.

With the exception of the N terminal 60 amino acids and the region between amino acid residues 200-230, human and mouse Tcte-1 proteins are highly conserved. It is of interest to note that both regions are highly hydrophilic, consistent with their presence on the cell surface and their role in species specific recognition.

7. EXAMPLE: TCTE-1 IS EXPRESSED IN A VARIETY OF SPECIES

Genomic DNA from cow, pig, dog, rabbit, guinea pig, human, monkey, chicken, Xenopus laevis, and zebra fish was digested with TaqI restriction enzyme and subjected to Southern blot analysis using [^{32}P]-labeled mouse Tcte-1 whole cDNA as a probe. Hybridization conditions were at 65°C, and washing was performed at 65°C in 0.1 x SSC. As shown in Figure 16, the Tcte-1 probe hybridized to distinct bands in DNA lanes corresponding to every species tested, indicating that tcte-protein is expressed by a variety of diverse species.

8. EXAMPLE: GENERATION OF A MONOCLONAL ANTIBODY DIRECTED TOWARD HUMAN TCTE-1

Mice were immunized with 50μg of TCTE-1 fusion protein as described in Section 6 supra, then were boosted after 4 weeks with 100μg TCTE-1 and, after another four weeks, with 25μg of TCTE-1. one week later, fusion to generate hybridomas was performed using standard techniques. Hybridoma supernatants were tested for the presence of anti-TCTE-1 antibody

by Western blot. As shown in Figure 17, the supernatant from hybridoma 4F7 bound selectively to the human TCTE-1 fusion protein.

9. EXAMPLE: IMMUNOFLUORESCENT LABELING OF HUMAN SPERM USING ANTI-TCTE-1 ANTIBODY

40 μl aliquots of sperm at a concentration of 100ng/μl were applied to enclosed circles on poly-L-lysine-coated slides, and allowed to attach for 20 min. Slides were fixed with 4% formaldehyde in PBS for 15 min., and washed with 100mM glycine followed by 1% normal goat serum in PBS. Anti-TCTE1 IgG or preimmune IgG was applied at a concentration of 0.1 μg/μl. Slides were washed again three times with PBS, treated with biotin-labelled goat anti-rabbit IgG (1:200), washed again, and treated with avidin-labelled FITC. After a final set of PBS washes, slides were rinsed with 10 mM Tris pH 7.5, allowed to dry at 37°C and mounted in glycerol. For accurate localization of FITC staining, color photography was performed with simultaneous epi-illumination with ultraviolet light (for FITC excitation) and trans-illumination with white light for an overlying phase constrast image. Figure 18 is a photograph of human sperm, showing immunofluorescent labelling of TCTE-1 protein.

10. DEPOSITS OF MICROORGANISMS

The following deposits have been made with the American Type Culture Collection, Rockville, MD.

(i) plasmid phTCTE1, deposited September 9, 1991, containing human TCTE-1 cDNA, assigned accession number 75095;

(ii) phage 1hTCTE1g, deposited September 9, 1991, containing human TCTE-1 genomic DNA, assigned accession number 75097;

(iii) plasmid pmTCTE1, deposited September 9, 1991, containing mouse Tcte-1 cDNA, assigned accession number 75096; and

(iv) hybridoma TCTE1-4F7, submitted October 1, 1992 and received October 2, 1992, assigned accession number _____.

Various references have been cited herein that are hereby incorporated by reference in their entirety.

64-1

Applicant's or agent's file reference number PCT-5.1	International application No.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 63-64, line

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit	Accession Number
September 9, 1991	75095

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
[X] This sheet was received with the international application	[ ] This sheet was received by the International Bureau on:
Authorized officer Nathaniel R. Hayden PCT INTERNATIONAL SERVICES DIVISION	Authorized officer

Form PCT/RO/134 (July 1992)

Form PCT/RO/134 continued Agent's File Reference No. PCT-551

AMERICAN TYPE CULTURE COLLECTION

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit	September 9, 1991	Accession Number	75096
Date of deposit	September 9, 1991	Accession Number	75097
Date of deposit	October 2, 1992	Accession Number	-

WHAT IS CLAIMED IS:

1. A substantially purified nucleic acid molecule of claim 1 having a sequence substantially as depicted in Fig. 3 for human TCTE-1.

2. A portion of the substantially purified nucleic acid molecule of claim 1 having a sequence substantially as depicted in Fig. 3 for the portion extending between about nucleotide 149 to about nucleotide 328, encoding the N-terminal 60 amino acid residues of TCTE-1 protein.

3. A portion of the substantially purified nucleic acid molecule of claim 1 having a sequence substantially as depicted in Fig. 3 for TCTE-1 for the portion extending between about nucleotide 329 to about nucleotide 1672.

4. A substantially purified nucleic acid molecule that encodes a human TCTE-1 protein having an amino acid sequence substantially as depicted in Fig. 5.

5. A substantially purified nucleic acid molecule having a sequence as contained in 1hTCTE1g as deposited with the American Type Culture Collection and having accession No. 75097.

6. The nucleic acid molecule of claim 1 or 4 comprised in a nucleic acid vector.

7. The nucleic acid molecule of claim 1 or 4 that is linked to a promoter sequence.

8. The nucleic acid molecule of claim 1 or 4 that is linked to at least a portion of another gene.

9. A microorganism into which has been introduced the nucleic acid molecule of claim 1 or 4.

10. A cell into which has been introduced the nucleic acid molecule of claim 1 or 4.

11. A transgenic animal into which has been introduced the nucleic acid molecule of claim 1 or 4.

12. Substantially purified human TCTE-1 protein.

13. The TCTE-1 protein of claim 12 having a sequence substantially as depicted in Fig. 5, or a functional equivalent, fragment, or derivative thereof.

14. The TCTE-1 protein of claim 12 having a sequence substantially as depicted in Fig. 5 for the portion extending between amino acids 1 and 60.

15. The TCTE-1 protein of claim 12 having a sequence substantially as depicted in Fig. 5 for the portion extending between amino acids 61 and 503.

16. A substantially purified protein comprising the TCTE-1 protein, fragment, or derivative of claim 13.

17. A substantially purified protein having (i) a molecular weight of about 50kD; and (ii) a homology of at least eighty percent to a corresponding length

of tcte-1 sequence, which is substantially identical to a 50kD protein found in sperm lysates.

18. A substantially purified protein having (i) a molecular weight of about 35kD; and (ii) a region of homology of at least 20 amino acids that are at least seventy-five percent homologous to a corresponding length of tcte-1 sequence, which is substantially identical to a 35KD protein found in liver and brain extracts.

19. A substantially purified antibody directed toward tcte-1.

20. The antibody of claim 19 that is directed toward human TCTE-1.

21. A vaccine comprising a tcte-1 protein.

22. The vaccine of claim 21 comprising the TCTE-1 protein of claim 12 or 13 in a suitable pharmaceutical carrier.

23. A vaccine comprising a nonpathogenic virus which carries a gene that encodes a tcte-1 protein or an immunogenic fragment thereof.

24. The vaccine of claim 23 in which the gene comprises a nucleic acid molecule according to claim 1 or 4.

25. A method of immunocontraception comprising administering to a subject in need of such treatment an effective amount of the vaccine of claim 21.

26. A method of immunocontraception comprising administering to a subject in need of such treatment an effective amount of the vaccine of claim 22.

27. A method of immunocontraception comprising administering to a subject in need of such treatment an effective amount of the vaccine of claim 23.

28. A method of immunocontraception comprising administering to a subject in need of such treatment an effective amount of the vaccine of claim 24.

29. A method of immunocontraception comprising administering to a subject in need of such treatment an effective amount of the antibody of claim 19.

30. A method of immunocontraception comprising administering to a subject in need of such treatment an effective amount of the antibody of claim 20.

31. A method for selecting sperm of a particular genotype comprising:

(i) producing a transgenic nonhuman animal of one species that carries a transgene encoding a tcte-1 protein from a second species of animal in which the transgene is located on the same chromosome as a gene of interest, such that a diploid cell of the transgenic animal contains only one copy of the chromosome that carries the transgene and the gene of interest;

(ii) collecting sperm from the transgenic animal;

(iii) exposing the sperm to a protein that binds to tcte-1 and is bound to a support that renders the protein retrievable, under conditions

that promote the binding of tcte-1 to the protein;

(iv) retrieving the protein bound to sperm; and

(v) releasing the sperm from the protein.

32. A method for improving fertility comprising augmenting the amount of tcte-1 expressed by sperm.

33. A method for producing an interspecies hybrid comprising mating a non-human animal of a first species to a non-human transgenic animal of a second species which carries, as a transgene, a gene that encodes a protein having the characteristics of a tcte-1 protein derived from the first species.

34. A monoclonal antibody that binds to TCTE-1.

35. The monoclonal antibody of claim 34 that is antibody 4F7, as produced by hybridoma 4F7.

36. A monoclonal antibody that is capable of competing with the antibody of claim 35 for binding to its target epitope.

37. A method of diagnosing infertility in a test subject comprising (i) quantifying the amount of tcte-1 protein expressed on sperm collected from the test subject; and (ii) comparing the amount of tcte-1 protein quantified in step (i) with the amount of tcte-1 protein expressed on sperm of a normal subject, wherein a difference in the amount of tcte-1 protein expressed on sperm of the test subject and the normal subject indicates that the test subject may be infertile.

38. The method of claim 37 in which the difference in the amount of tcte-1 protein in the test subject relative to the normal subject is that the amount of tcte-1 protein expressed by the test subject is less than 70 percent of the amount of tcte-1 expressed by the normal subject.

39. The method of claim 37 in which the difference in the amount of tcte-1 protein in the test subject relative to the normal subject is that the amount of tcte-1 protein expressed by the test subject is less than 50 percent of the amount of tcte-1 expressed by the normal subject.

40. The method of claim 37 in which the difference in the amount of tcte-1 protein in the test subject relative to the normal subject is that the amount of tcte-1 protein expressed by the test subject is less than 25 percent of the amount of tcte-1 expressed by the normal subject.

FIGURE 1

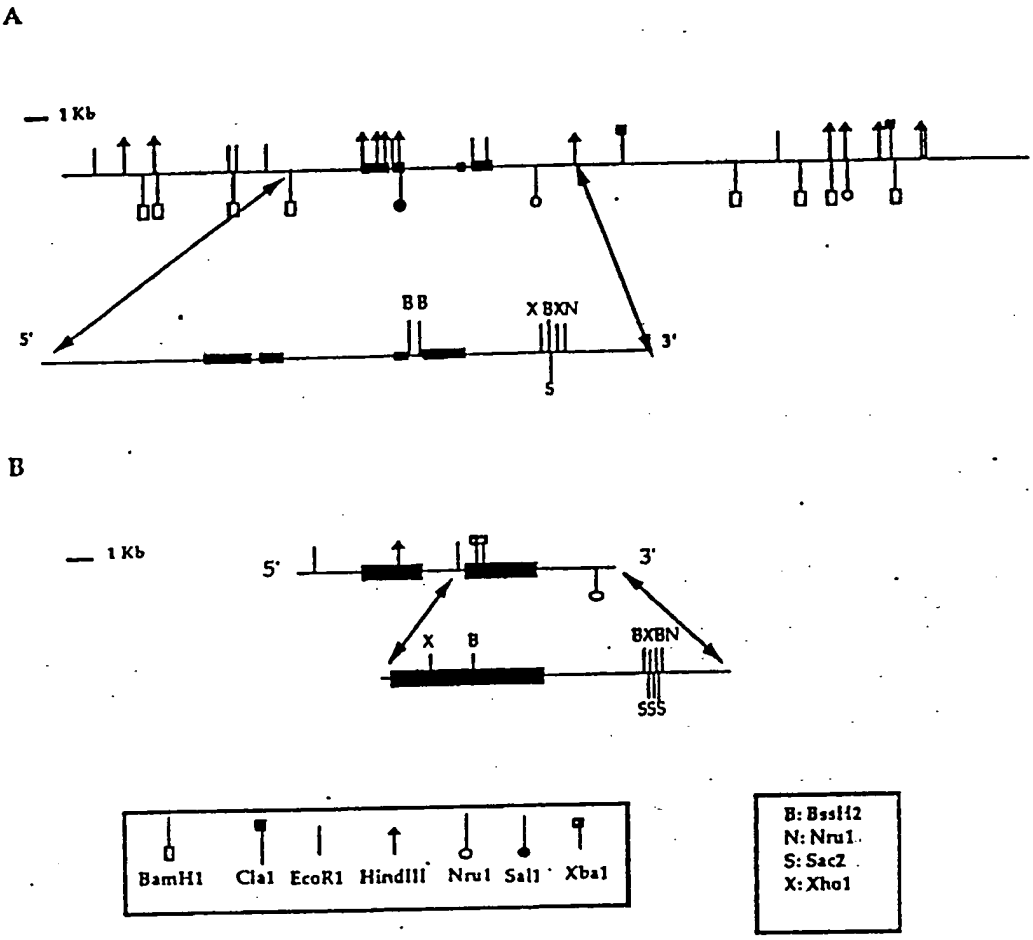
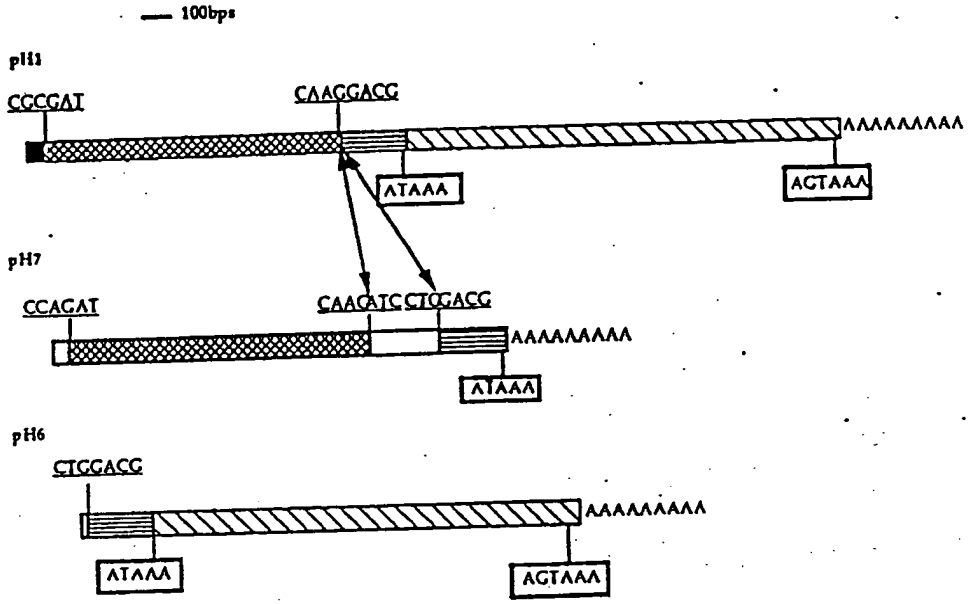

A
1 Kb
5'
BB
XBXN
3'
S
B
1 Kb
5'
3'
X
B
BXBN
SSS
BamH1
Cla1
EcoR1
HindIII
Nru1
Sal1
Xba1
B: BssH2
N: Nru1
S: Sac2
X: Xho1

FIGURE 2


100bps
pH1
CGCGAT
CAAGGACG
AAAAAAAAA
ATAAA
AGTAAA
pH7
CCAGAT
CAAGATC CTGGACG
AAAAAAAAA
ATAAA
pH6
CTGGACG
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ATAAA
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FIGURE 3

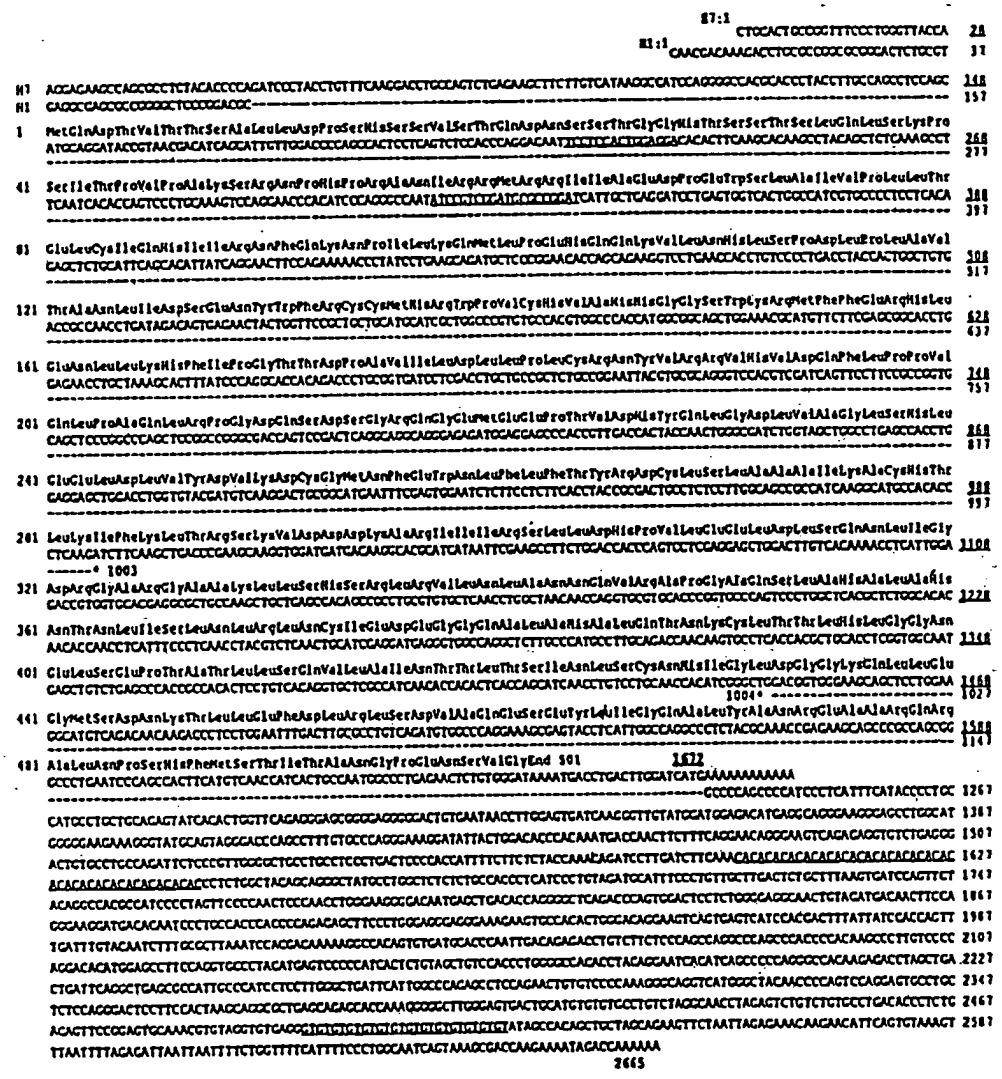


FIGURE 4

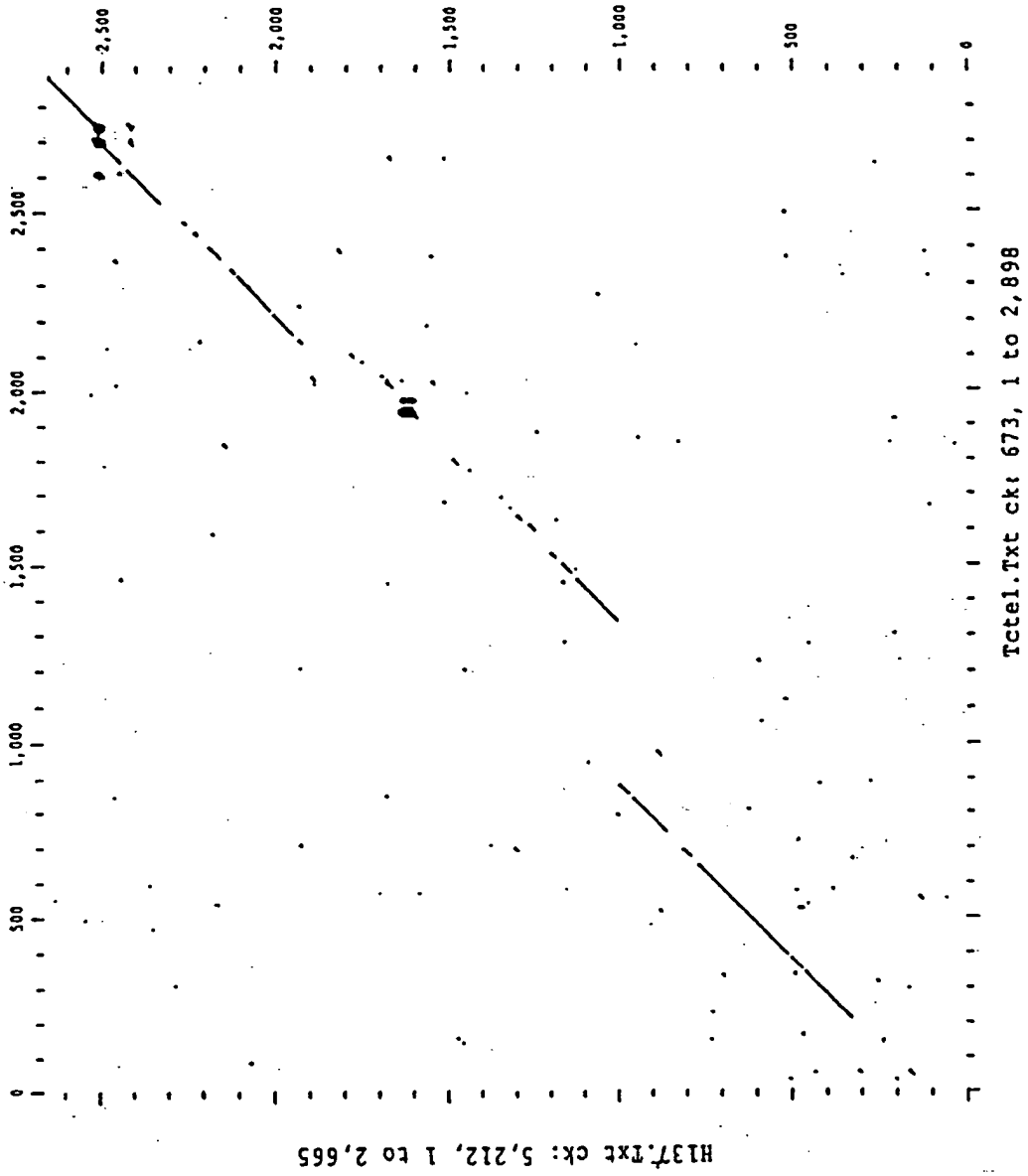

2,500
2,000
1,500
1,000
500
0
Tctel.Txt ck: 673, 1 to 2,898
H13T.Txt ck: 5,212, 1 to 2,665

FIGURE 5

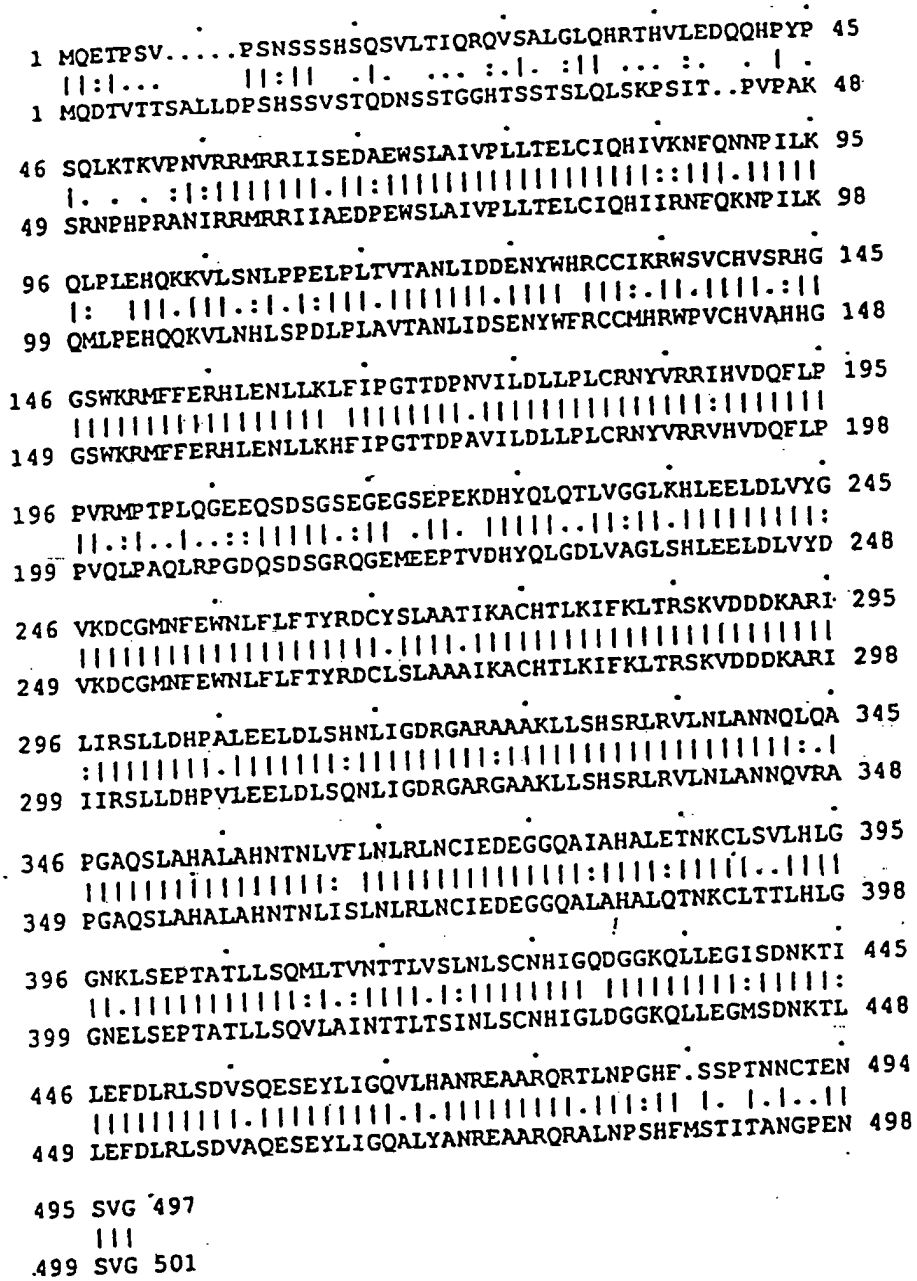
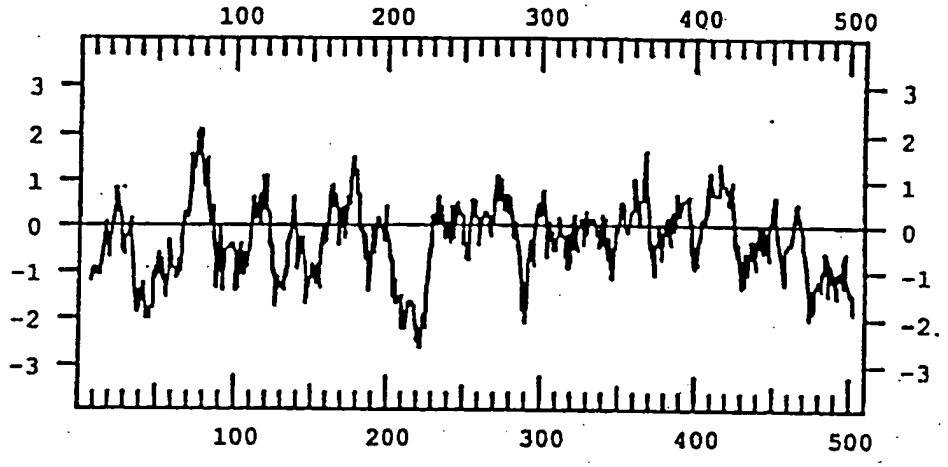

1 MQETPSV.....PSNSSSHSQSVLTIQRQVSALGLQHRTHVLEDQQHPYP 45
1 MQDTVTTSALLDPSHSSVSTQDNSSTGGHTSSTSLQLSKPSIT..PVPAK 48
46 SQLKTKVPNVRRMRRIISEDAEWSLAIVPLLTELCIQHIVKNFQNNPILK 95
49 SRNPHPRANIRRMRRIIAEDPEWSLAIVPLLTELCIQHIIRNFQKNPILK 98
96 QLPLEHQKKVLSNLPPELPLTVTANLIDDENYWHRCCIKRWSVCHVSRHG 145
99 QMLPEHQQKVLNHLSPDLPLAVTANLIDSENYWFRCCMHRWPVCHVAHHG 148
146 GSWKRMFFERHLENLLKLFIPGTTDPNVILDLLPLCRNYVRRIHVDQFLP 195
149 GSWKRMFFERHLENLLKHFIPGTTDPAVILDLLPLCRNYVRRVHVDQFLP 198
196 PVRMPTPLQGEEQSDSGSEGEGSEPEKDHYQLQTLVGGLKHLEELDLVYG 245
199 PVQLPAQLRPGDQSDSGRQGEMEEPTVDHYQLGDLVAGLSHLEELDLVYD 248
246 VKDCGMNFEWNLFLFTYRDCYSLAATIKACHTLKIFKLTRSKVDDDKARI 295
249 VKDCGMNFEWNLFLFTYRDCLSLAAAIKACHTLKIFKLTRSKVDDDKARI 298
296 LIRSLLDHPALEELDLSHNLIGDRGARAAAKLLSHSRLRVLNLANNQLQA 345
299 IIRSLLDHPVLEELDLSQNLIGDRGARGAAKLLSHSRLRVLNLANNQVRA 348
346 PGAQSLAHALAHNTNLVFLNLRLNCIEDEGGQAIAHALETNKCLSVLHLG 395
349 PGAQSLAHALAHNTNLISLNLRLNCIEDEGGQALAHALQTNKCLTTLHLG 398
396 GNKLSEPTATLLSQMLTVNTTLVSLNLSCNHIGQDGGKQLLEGISDNKTI 445
399 GNELSEPTATLLSQVLAINTTLTSINLSCNHIGLDGGKQLLEGMSDNKTL 448
446 LEFDLRLSDVSQESEYLIGQVLHANREAARQRTLNPGHF.SSPTNNCTEN 494
449 LEFDLRLSDVAQESEYLIGQALYANREAARQRALNPSHFMSTITANGPEN 498
495 SVG 497
499 SVG 501

FIGURE 6A-B

A



B

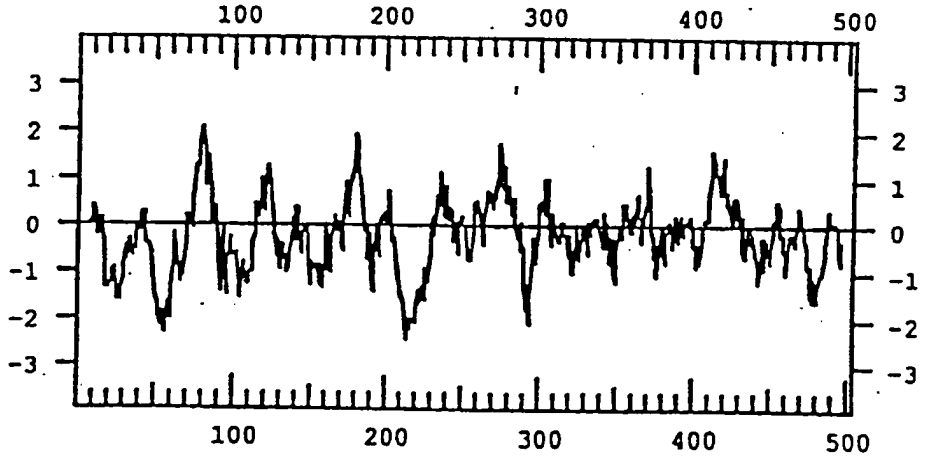
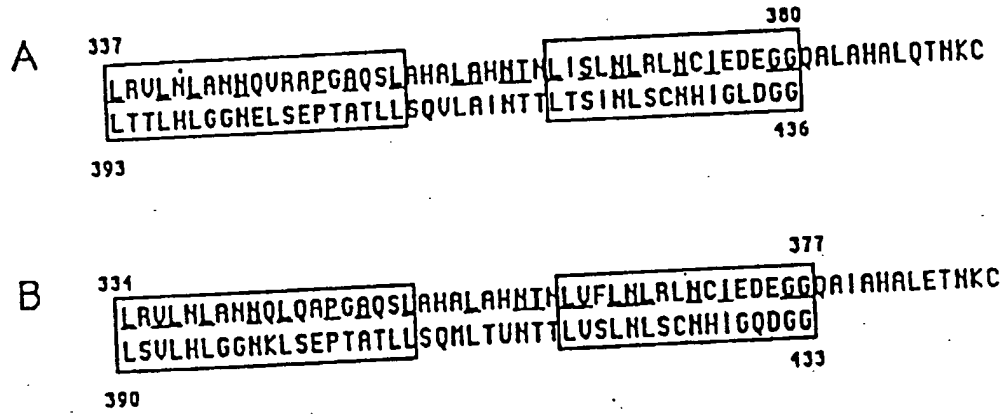


FIGURE 7 A-B


A
337
380
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LTTLHLGGNELSEPTATLLSQVLAINTTLTSINLSCNHIGLDGG
393
436
B
334
377
LRVLNLANNQLQAPGAQSLAHALAHNINLVFLNLRLNCIEDEGGDAIAHALETNKC
LSVLHLGGNKLSEPTATLLSQMLTUNTTLVSLNLSCNHIGQDGG
390
433

FIGURE 8 A-C

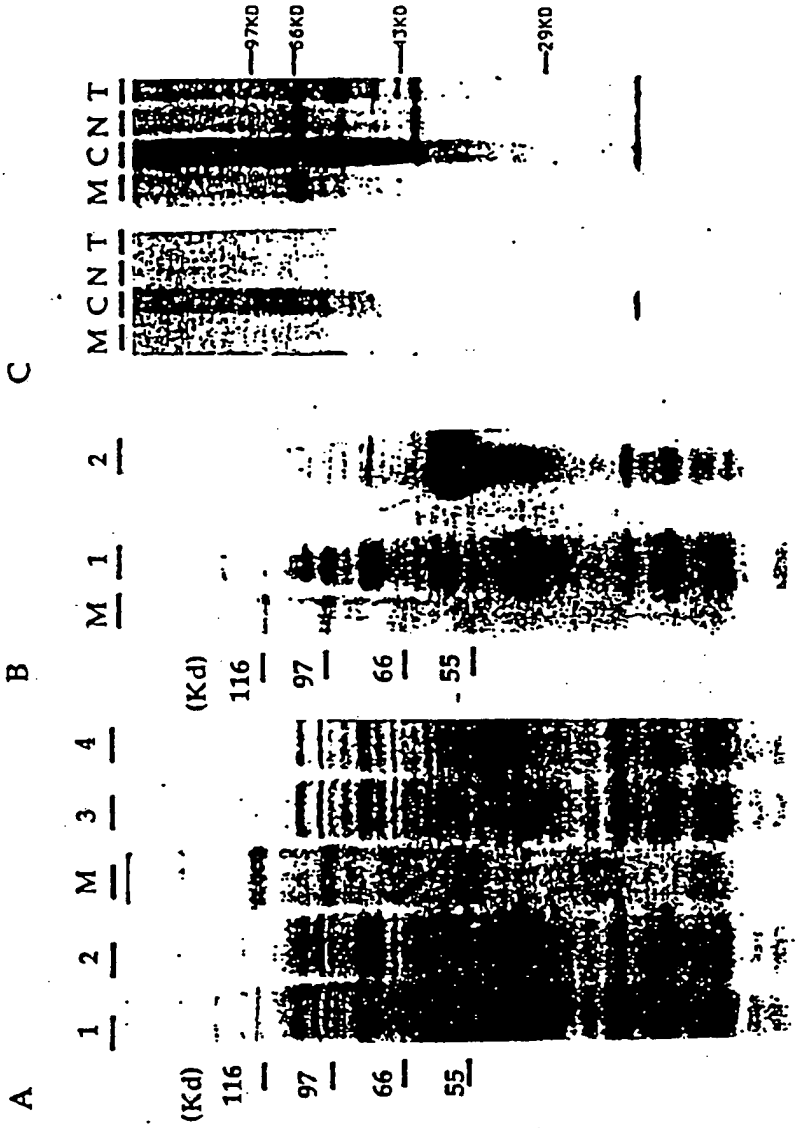

C
M C N T
M C N T
97KD
66KD
43KD
29KD
B
M 1 2
(Kd)
116
97
66
55
A
1 2 M 3 4
(Kd)
116
97
66
55

FIGURE 9 A-B

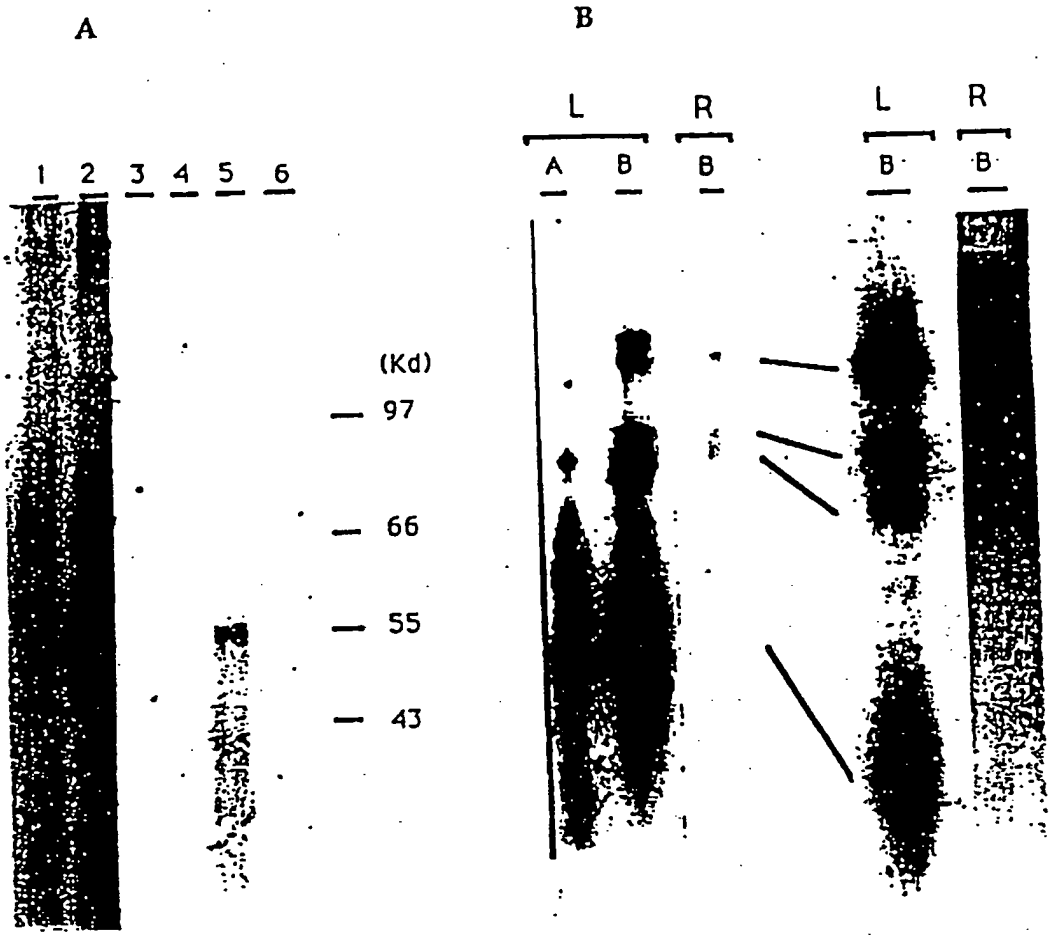


FIGURE 10

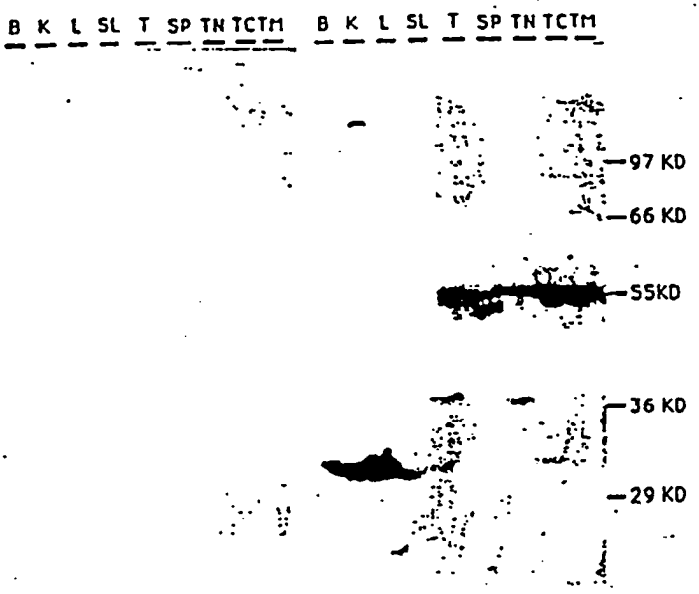
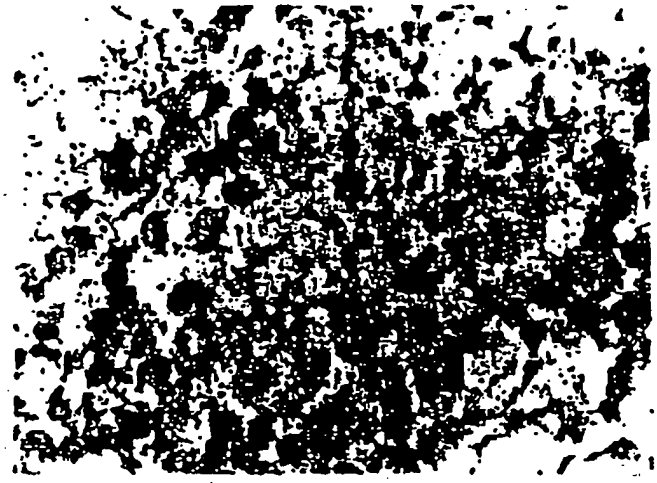


FIGURE 11

AS



S

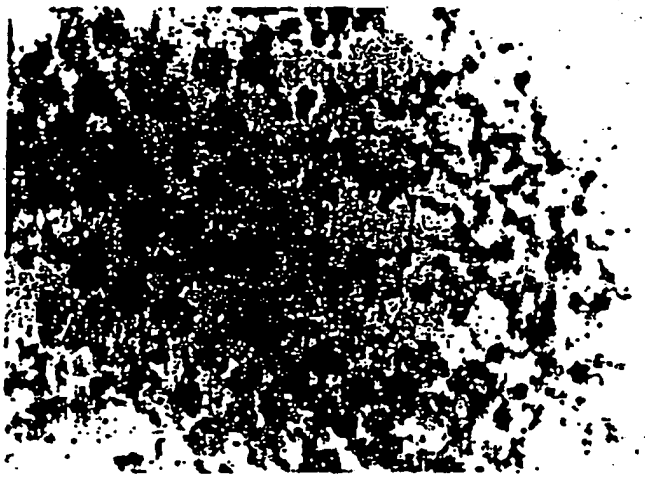


FIGURE 12 A-C

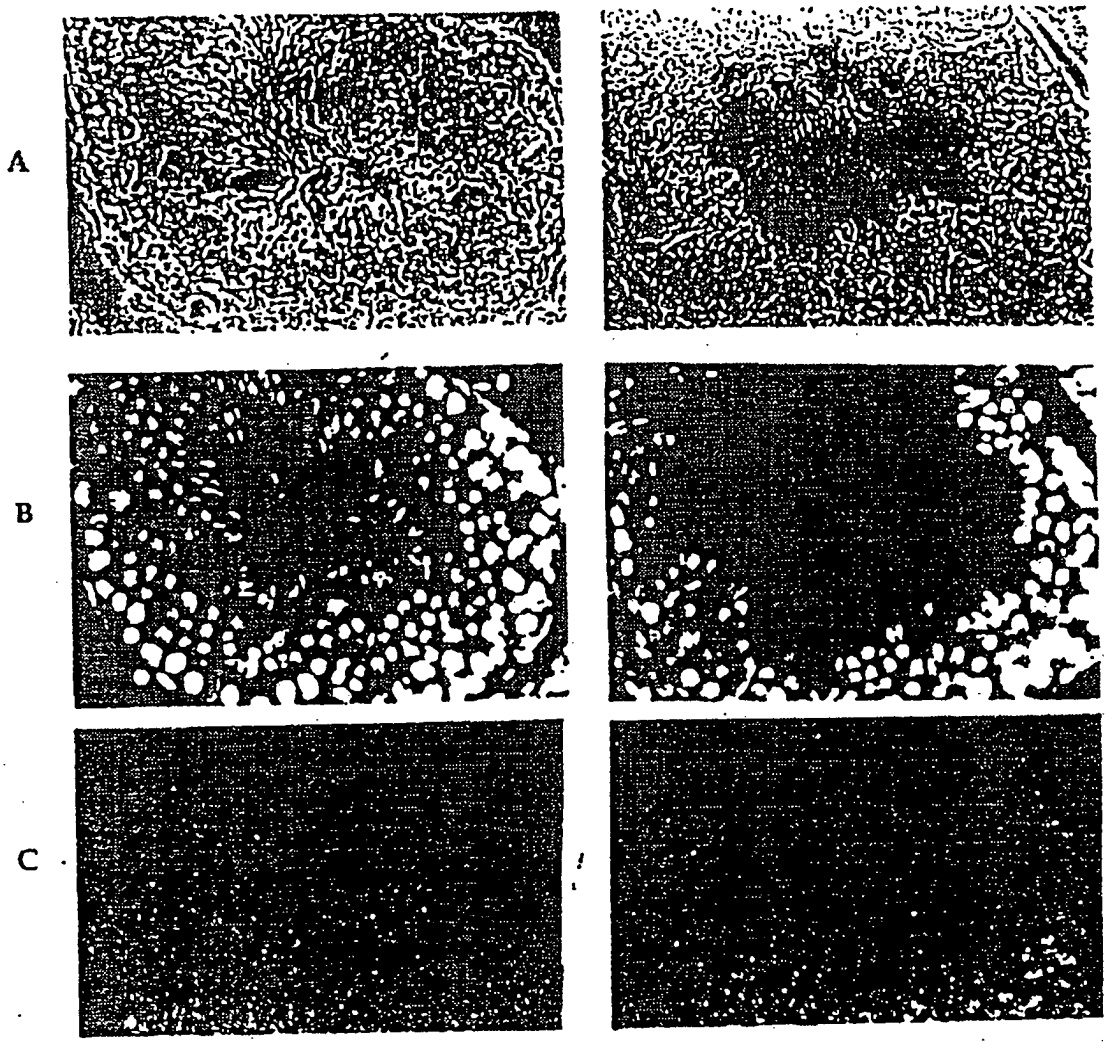


FIGURE 13 A-C

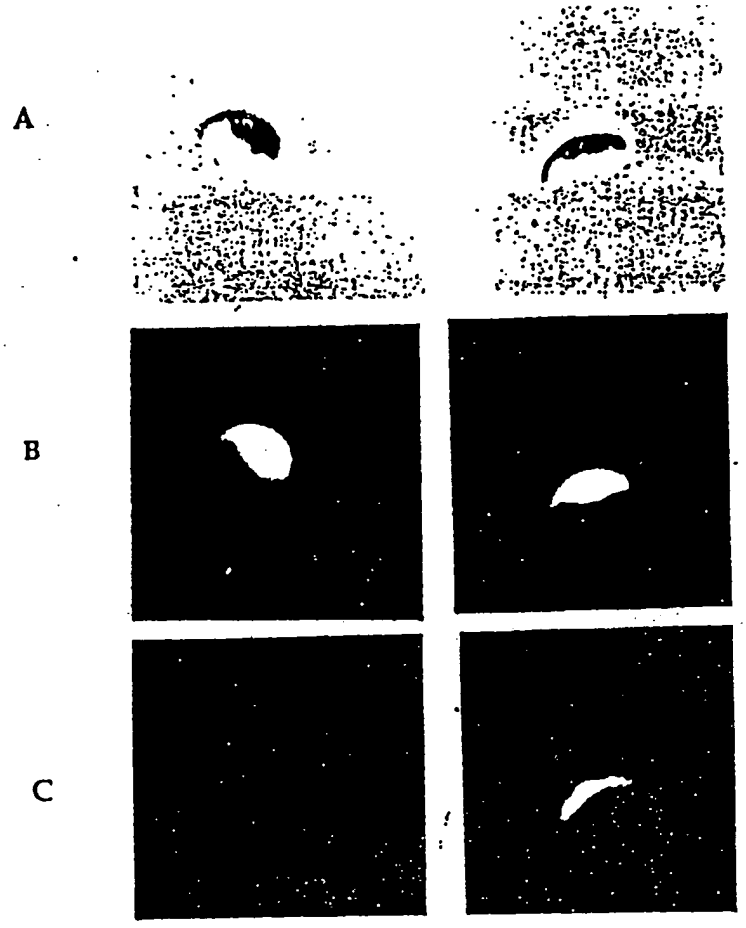
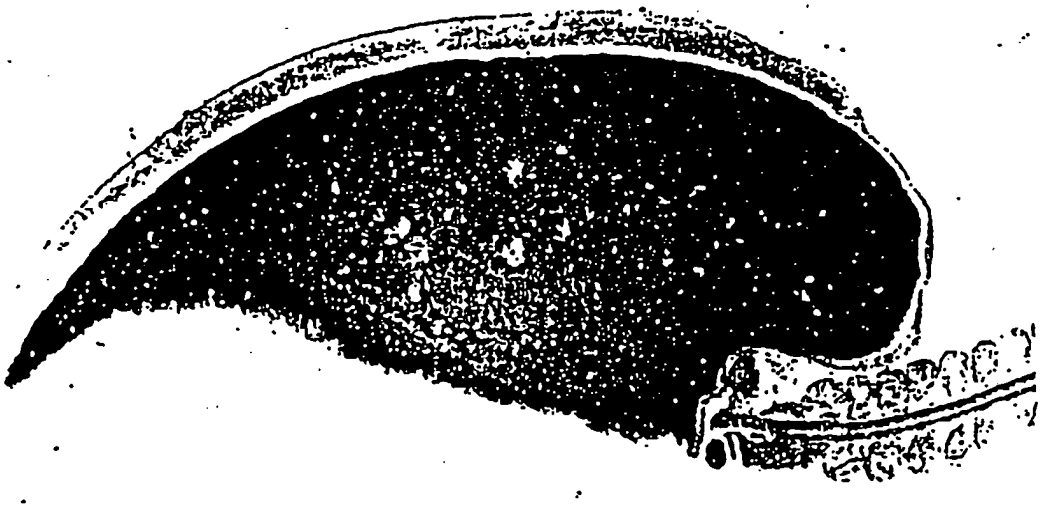


FIGURE 14 A-B

A

B



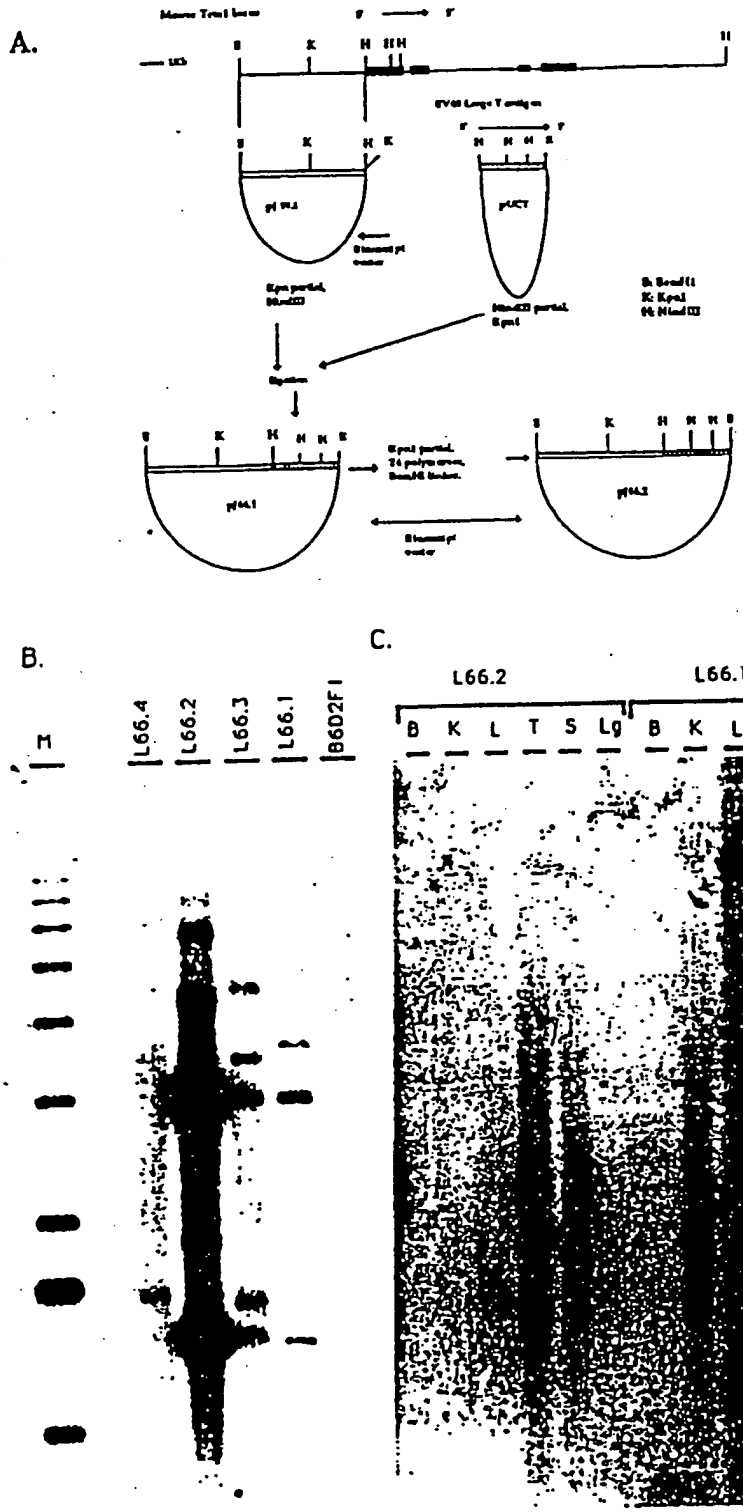
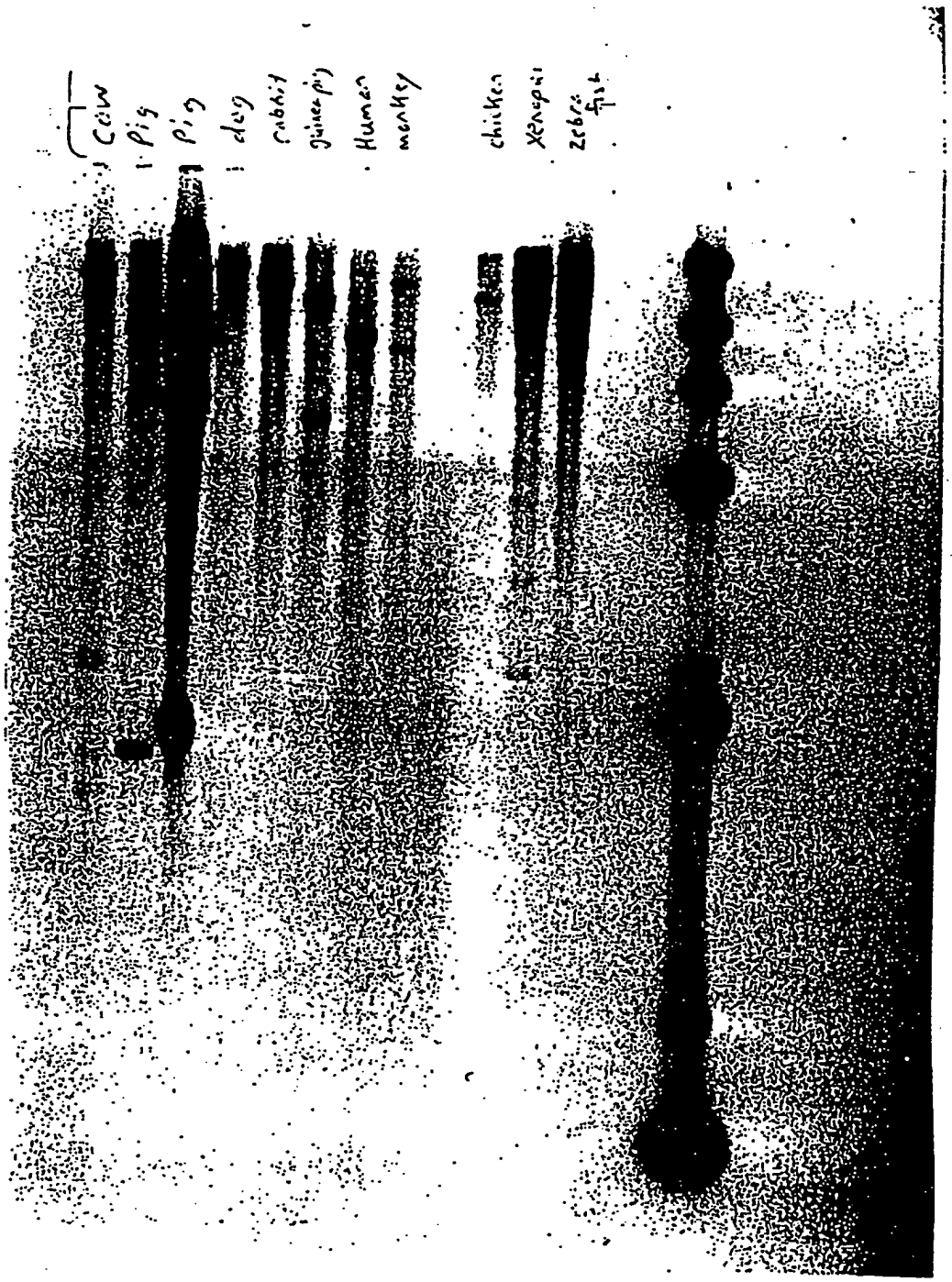


FIGURE 15 A-C

FIGURE 16.


Cow
Pig
Pig
dog
rabbit
guinea pig
Human
monkey
chicken
Xenopus
zebra fish

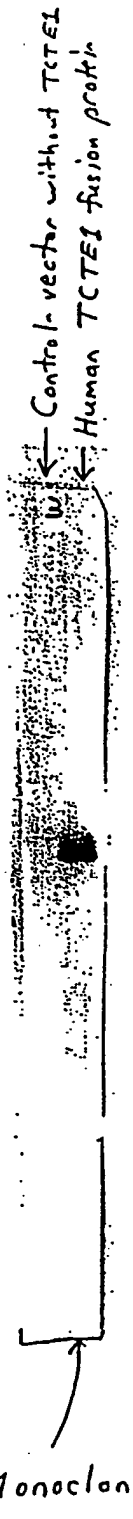
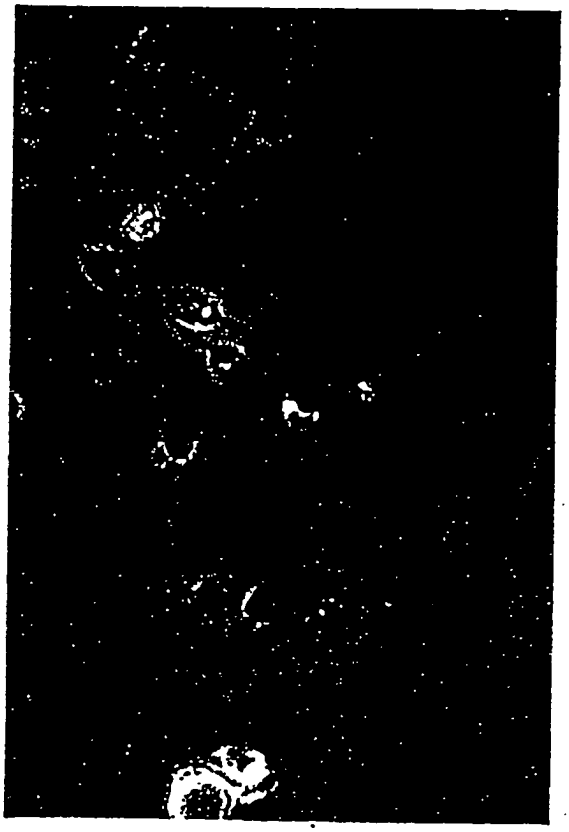

Control vector without TCTEI
Human TCTEI fusion protein
Monoclonal no. 4F7 (IgG2a)

FIGURE 17.

FIGURE 18.



INTERNA[illegible]NAL SEARCH REPORT

[illegible]tional application No.
US92/08457

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 85.8; 435/69.1, 172.3, 252.3, 240.2; 514/2; 530/388.1, 387.1, 388.4, 389.1; 536/267, 800.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CA, WPI, APS
search terms: Lee Silver, TCTE-1, D17Si11, immunocontraception, sperm, infertility

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Immunogenetics, Volume 30, issued June 1989, N. Sarvetnick et al., "A mouse chromosome 17 gene encodes a testes-specific transcript with unusual properties", pages 34-41, see entire document.	1-40
Y	Genomics, Volume 5, issued June 1989, K.B. Bibbins et al., "Human homologs of two testes-expressed loci on mouse chromosome 17 map to opposite arms of chromosome 6, pages 139-143, see entire document.	1-40
Y	Nature, Volume 335, issued 06 October 1988, P. Primakoff et al., "Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20", pages 543-546, see entire document.	12-40
Y	USA 4,870,009 (Evans et al.) 26 September 1989, see entire document.	1[illegible],31,33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 DECEMBER 1992	12 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer PHILLIP GAMBEL Telephone No. (703) 308-0196

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INTERNAT L SEARCH REPORT

ional application No.
PCT/US92/08457

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 39/00, 39/395; C07H 21/04; C07K 3/00, 13/00, 15/28, 17/00; C12N 15/00, 15/09; C12P 21/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/88, 85.8; 435/69.1, 172.3, 252.3, 240.2; 514/2; 530/388.1, 387.1, 388.4, 389.1; 536/267, 800/2